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PROTEASE INHIBITOR PEPTIDES

Background of the Invention

The plasma, or serine, proteases of the blood contact system are known to be activated by interaction with negatively charged surfaces. For example, tissue injury during surgery exposes the vascular basement membrane, causing interaction of the blood with collagen, which is negatively charged at physiological Ph. This induces a cascade of proteolytic events, leading to production of plasmin, a fibrinolytic protease, and consequent blood loss.

Perioperative blood loss of this type can be particularly severe during cardiopulmonary bypass (CPB) surgery, in which the patient's blood flow is diverted to an artificial heart-lung machine. CPB is an essential component of a number of life-saving surgical procedures. For example, in the United States, it is estimated that 300,000 patients every year undergo coronary artery bypass grafts involving the use of CPB.

Although necessary and generally safe, CPB is associated with a significant rate of morbidity, some of which may be attributed to a "whole body inflammatory response" caused by activation of plasma protease systems and blood cells through interactions with the artificial surfaces of the heart-lung machine (Butler *et al.*, *Ann. Thorac. Surg.* 55:552 (1993); Edmunds *et al.*, *J. Card. Surg.* 8:404 (1993)). For example, during extracorporeal circulation, exposure of blood to negatively charged surfaces of the artificial bypass circuit, e.g., plastic surfaces in the heart-lung machine, results in direct activation of plasma factor XII.

Factor XII is a single-chain 80 kDa protein that circulates in plasma as an inactive zymogen. Contact with negatively charged nonendothelial surfaces, like those of the bypass circuit, causes surface-bound factor XII to be autoactivated to the active serine protease factor XIIa. See Colman, *Agents Actions Suppl.* 42:125

(1993). Surface-activated factor XIIa then processes prekallikrein (PK) to active kallikrein, which in turn cleaves more XIIa from XII in a reciprocal activation reaction that results in a rapid amplification of the contact pathway. Factor XIIa can also activate the first component of complement C1, leading to production of the anaphylatoxin C5a through the classical complement pathway.

The CPB-induced inflammatory response includes changes in capillary permeability and interstitial fluid accumulation. Cleavage of high molecular weight kininogen (HK) by activated kallikrein generates the potent vasodilator bradykinin, which is thought to be responsible for increasing vascular permeability, resulting in edema, especially in the lung. The lung is particularly susceptible to damage associated with CPB, with some patients exhibiting what has been called "pump lung syndrome" following bypass, a condition indistinguishable from adult respiratory distress. See Johnson *et al.*, *J. Thorac. Cardiovasc. Surg.* 107:1193 (1994).

Post-CPB pulmonary injury includes tissue damage thought to be mediated by neutrophil sequestration and activation in the microvasculature of the lung. (Butler *et al.*, *supra*; Johnson, *et al.*, *supra*). Activated factor XII can itself stimulate neutrophil aggregation. Factor XIIa-generated kallikrein, and complement protein C5a generated by Factor XIIa activation of the complement cascade, both induce neutrophil chemotaxis, aggregation and degranulation. See Edmunds *et al.*, *supra* (1993). Activated neutrophils may damage tissue through release of oxygen-derived free-radicals, proteolytic enzymes such as elastase, and metabolites of arachidonic acid. Release of neutrophil products in the lung can cause changes in vascular tone, endothelial injury and loss of vascular integrity.

Intrinsic inhibition of the contact system occurs through inhibition of activated XIIa by C1-inhibitor (C1-INH). See Colman, *supra*. During CPB, this natural

inhibitory mechanism is overwhelmed by massive activation of plasma proteases and consumption of inhibitors. A potential therapeutic strategy for reducing post-bypass pulmonary injury mediated by neutrophil activation would, therefore, be to block the formation and activity of the neutrophil agonists kallikrein, factor XIIa, and C5a by inhibition of proteolytic activation of the contact system.

Protease inhibitor therapy which partially attenuates the contact system is currently employed clinically in CPB. Aprotinin, also known as basic pancreatic protease inhibitor (BPPI), is a small, basic, 58 amino acid polypeptide isolated from bovine lung. It is a broad spectrum serine protease inhibitor of the Kunitz type, and was first used during bypass in an attempt to reduce the inflammatory response to CPB. See Butler *et al.*, *supra*. Aprotinin treatment results in a significant reduction in blood loss following bypass, but does not appear to significantly reduce neutrophil activation. Additionally, since aprotinin is of bovine origin, there is concern that repeated administration to patients could lead to the development of an immune response to aprotinin in the patients, precluding its further use.

The proteases inhibited by aprotinin during CPB appear to include plasma kallikrein and plasmin. (See, e.g., Scott, *et al.*, *Blood* 69:1431 (1987)). Aprotinin is an inhibitor of plasmin (K_i of 0.23nM), and the observed reduction in blood loss may be due to inhibition of fibrinolysis through the blocking of plasmin action. Although aprotinin inhibits plasma kallikrein, (K_i of 20nM), it does not inhibit activated factor XII, and consequently only partially blocks the contact system during CPB.

Another attractive protease target for use of protease inhibitors, such as those of the present invention, is factor XIIa, situated at the very first step of contact activation. By inhibiting the proteolytic activity of factor XIIa, kallikrein production would be prevented, blocking amplification of

the contact system, neutrophil activation and bradykinin release. Inhibition of XIIa would also prevent complement activation and production of C5a. More complete inhibition of the contact system during CPB could, therefore, be achieved through the use of a better XIIa inhibitor.

Protein inhibitors of factor XIIa are known. For example, active site mutants of α_1-antitrypsin that inhibit factor XIIa have been shown to inhibit contact activation in human plasma. See Patston *et al.*, *J. Biol. Chem.* 265:10786 (1990). The large size and complexity (greater than 400 amino acid residues) of these proteins present a significant challenge for recombinant protein production, since large doses will almost certainly be required during CPB. For example, although it is a potent inhibitor of both kallikrein and plasmin, nearly 1 gram of aprotinin must be infused into a patient to inhibit the massive activation of the kallikrein-kinin and fibrinolytic systems during CPB.

The use of smaller, more potent XIIa inhibitors such as the corn and pumpkin trypsin inhibitors (Wen, *et al.*, *Protein Exp. & Purif.* 4:215 (1993); Pedersen, *et al.*, *J. Mol. Biol.* 236:385 (1994)) could be more cost-effective than the large α_1-antitrypsins, but the infusion of high doses of these non-mammalian inhibitors could result in immunologic reactions in patients undergoing repeat bypass operations. The ideal protein XIIa inhibitor is, therefore, preferably, small, potent, and of human sequence origin.

One candidate for an inhibitor of human origin is found in circulating isoforms of the human amyloid β-protein precursor (APPI), also known as protease nexin-2. APPI contains a Kunitz serine protease inhibitor domain known as KPI (Kunitz Protease Inhibitor). See Ponte *et al.*, *Nature*, 331:525 (1988); Tanzi *et al.*, *Nature* 331:528 (1988); Johnstone *et al.*, *Biochem. Biophys. Res. Commun.* 163:1248 (1989); Oltersdorf *et al.*, *Nature* 341:144 (1989). Human KPI shares about 45% amino acid sequence identity with aprotinin. The isolated KPI domain has

been prepared by recombinant expression in a variety of systems, and has been shown to be an active serine protease inhibitor. See, for example, Sinha, *et al.*, *J. Biol. Chem.* 265:8983 (1990). The measured *in vitro* K_i of KPI against plasma kallikrein is 45nM, compared to 20nM for aprotinin.

Aprotinin, KPI, and other Kunitz-type serine protease inhibitors have been engineered by site-directed mutagenesis to improve inhibitory activity or specificity. Thus, substitution of Lys^{15} of aprotinin with arginine resulted in an inhibitor with a K_i of 0.32nM toward plasma kallikrein, a 100-fold improvement over natural aprotinin. See PCT application No. 89/10374. See also Norris *et al.*, *Biol. Chem. Hoppe Seyler* 371:3742 (1990). Alternatively, substitution of position 15 of aprotinin with valine or substitution of position 13 of KPI with valine resulted in elastase inhibitors with K_is in the 100 pM range, although neither native aprotinin nor native KPI significantly inhibits elastase. See Wenzel *et al.*, in: *Chemistry of Peptides and Proteins, Vol. 3*, (Walter de Gruyter, Berlin, New York, 1986); Sinha *et al.*, *supra*. Methods for substituting residues 13, 15, 37, and 50 of KPI are shown in general terms in European Patent Application No. 0 393 431, but no specific sequences are disclosed, and no protease inhibition data are given.

Phage display methods have been recently used for preparing and screening derivatives of Kunitz-type protease inhibitors. See PCT Application No. 92/15605, which describes specific sequences for 34 derivatives of aprotinin, some of which were reportedly active as elastase and cathepsin inhibitors. The amino acid substitutions in the derivatives were distributed throughout almost all positions of the aprotinin molecule.

Phage display methods have also been used to generate KPI variants that inhibit factor VIIa and kallikrein. See Dennis *et al.*, *J. Biol. Chem.* 269:22129 and 269:22137 (1994). The residues that could be varied in the phage

display selection process were limited to positions 9-11, 13-17, 32, 36 and 37, and several of those residues were also held constant for each selection experiment. One of those variants was said to have a K_i of 1.2nM for kallikrein, and had substitutions at positions 9 (Thr→Pro), 13 (Arg→Lys), 15 (Met→Leu), and 37 (Gly→Tyr). None of the inhibitors was tested for the ability to inhibit factor XIIa.

It is apparent, therefore, that new protease inhibitors that can bind to and inhibit the activity of serine proteases are greatly to be desired. In particular it is highly desirable to prepare peptides, based on human peptide sequences, that can inhibit selected serine proteases such as kallikrein; chymotrypsins A and B; trypsin; elastase; subtilisin; coagulants and procoagulants, particularly those in active form, including coagulation factors such as factors VIIa, IXa, Xa, XIa, and XIIa; plasmin; thrombin; proteinase-3; enterokinase; acrosin; cathepsin; urokinase; and tissue plasminogen activator. It is also highly desirable to prepare novel protease inhibitors that can ameliorate one or more of the undesirable clinical manifestations associated with enhanced serine protease activity, for example by reducing pulmonary damage or blood loss during CPB.

Summary of the Invention

The present invention relates to peptides that can bind to and preferably exhibit inhibition of the activity of serine proteases. Those peptides can also provide a means of ameliorating, treating or preventing clinical conditions associated with increased activity of serine proteases. Particularly, the novel peptides of the present invention preferably exhibit a more potent and specific (i.e., greater) inhibitory effect toward serine proteases of interest in comparison to known serine protease inhibitors. Examples of such proteases include: kallikrein; chymotrypsins A and B; trypsin; elastase; subtilisin; coagulants and procoagulants, particularly

those in active form, including coagulation factors such as factors VIIa, IXa, Xa, XIa, and XIIa; plasmin; thrombin; proteinase-3; enterokinase; acrosin; cathepsin; urokinase; and tissue plasminogen activator.

In achieving the inhibition of serine protease activity, the invention provides protease inhibitors that can ameliorate one or more of the undesirable clinical manifestations associated with enhanced serine protease activity, for example, by reducing pulmonary damage or blood loss during CPB.

The present invention relates to protease inhibitors comprising the following amino acid sequences (SEQ ID NO:1): X^1-Val-Cys-Ser-Glu-Gln-Ala-Glu-X^2-Gly-X^3-Cys-Arg-Ala-X^4-X^5-X^6-X^7-Trp-Tyr-Phe-Asp-Val-Thr-Glu-Gly-Lys-Cys-Ala-Pro-Phe-X^8-Tyr-Gly-Gly-Cys-X^9-X^{10}-X^{11}-X^{12}-Asn-Asn-Phe-Asp-Thr-Glu-Glu-Tyr-Cys-Met-Ala-Val-Cys-Gly-Ser-Ala-Ile,

wherein: X^1 is selected from (SEQ ID NO:2) Glu-Val-Val-Arg-Glu-, Asp, or Glu; X^2 is selected from Thr, Val, Ile and Ser; X^3 is selected from Pro and Ala; X^4 is selected from Arg, Ala, Leu, Gly, or Met; X^5 is selected from Ile, His, Leu, Lys, Ala, or Phe; X^6 is selected from Ser, Ile, Pro, Phe, Tyr, Trp, Asn, Leu, His, Lys, or Glu; X^7 is selected from Arg, His, or Ala; X^8 is selected from Phe, Val, Leu, or Gly; X^9 is selected from Gly, Ala, Lys, Pro, Arg, Leu, Met, or Tyr; X^{10} is selected from Ala, Arg, or Gly; X^{11} is selected from Lys, Ala, or Asn; and X^{12} is selected from Ser, Ala, or Arg.

The invention relates more specifically to protease inhibitors comprising the following amino acid sequences (SEQ ID NO:1):

X^1-Val-Cys-Ser-Glu-Gln-Ala-Glu-X^2-Gly-X^3-Cys-Arg-Ala-X^4-X^5-X^6-X^7-Trp-Tyr-Phe-Asp-Val-Thr-Glu-Gly-Lys-Cys-Ala-Pro-Phe-X^8-Tyr-Gly-Gly-Cys-X^9-X^{10}-X^{11}-X^{12}-Asn-Asn-Phe-Asp-Thr-Glu-Glu-Tyr-Cys-Met-Ala-Val-Cys-Gly-Ser-Ala-Ile,

wherein X^1 is selected from (SEQ ID NO:2) Glu-Val-Val-Arg-Glu-, Asp, or Glu; X^2 is selected from Thr, Val, Ile

and Ser; X^3 is selected from Pro and Ala; X^4 is selected from Arg, Ala, Leu, Gly, or Met; X^5 is selected from Ile, His, Leu, Lys, Ala, or Phe; X^6 is selected from Ser, Ile, Pro, Phe, Tyr, Trp, Asn, Leu, His, Lys, or Glu; X^7 is selected from Arg, His, or Ala; X^8 is selected from Phe, Val, Leu, or Gly; X^9 is selected from Gly, Ala, Lys, Pro, Arg, Leu, Met, or Tyr; X^{10} is selected from Ala, Arg, or Gly; X^{11} is selected from Lys, Ala, or Asn; X^{12} is selected from Ser, Ala, or Arg; provided that when X^4 is Arg, X^6 is Ile; when X^9 is Arg, X^4 is Ala or Leu; when X^9 is Tyr, X^4 is Ala or X^5 is His; and either X^5 is not Ile; or X^6 is not Ser; or X^9 is not Leu, Phe, Met, Tyr, or Asn; or X^{10} is not Gly; or X^{11} is not Asn; or X^{12} is not Arg.

Another aspect of this invention provides protease inhibitors wherein at least two amino acid residues selected from the group consisting of X^4, X^5, X^6, and X^7 defined above differ from the residues found in the naturally occurring sequence of KPI. Another aspect of this invention provides protease inhibitors wherein X^1 is Asp or Glu, X^2 is Thr, X^3 is Pro, and X^{12} is Ser. Yet another aspect of this invention provides protease inhibitors wherein X^1 is Glu, X^2 is Thr, X^3 is Pro, X^4 is Met, X^5 is Ile, X^6 is Ser, X^7 is Arg, x^8 is Phe, X^9 is Gly, X^{10} is Gly, and X^{11} is Asn. Another aspect of this invention provides protease inhibitors wherein X^1 is Asp, X^2 is Thr, X^3 is Pro, X^4 is Arg, X^5 is Ile, X^6 is Ile, X^7 is Arg, x^8 is Val, X^9 is Arg, X^{10} is Ala, and X^{11} is Lys. Another aspect of this invention provides protease inhibitors wherein X^1 is (SEQ ID NO:2) Glu-Val-Val-Arg-Glu-, X^2 is Thr, X^3 is Pro, X^4 is Met, X^5 is Ile, X^6 is Ser, X^7 is Arg, x^8 is Phe, X^9 is Gly, X^{10} is Gly, X^{11} is Asn, and X^{12} is Ala. Another aspect of this invention provides protease inhibitors wherein X^1 is (SEQ ID NO:2) Glu-Val-Val-Arg-Glu-, X^2 is Thr, X^3 is Pro, X^4 is Met, X^5 is Ile, X^6 is Ser, X^7 is Arg, x^8 is Phe, X^9 is Gly, X^{10} is Gly, X^{11} is Ala, and X^{12} is Arg. Another aspect of this invention provides protease inhibitors wherein X^1 is Glu, X^2 is Thr, X^3 is Pro, X^4 is Met, X^5 is Ile, X^6 is Ser, X^7 is

Arg, x^8 is Phe, X^9 is Gly, X^{10} is Ala, X^{11} is Asn, and X^{12} is Arg. Another aspect of this invention provides protease inhibitors wherein X^1 is (SEQ ID NO:2) Glu-Val-Val-Arg-Glu-, X^2 is Thr, X^3 is Pro, X^4 is Met, X^5 is Ile, X^6 is Ser, X^7 is Arg, x^8 is Phe, X^9 is Gly, X^{10} is Arg, X^{11} is Asn, and X^{12} is Arg. Another aspect of this invention provides protease inhibitors wherein X^1 is (SEQ ID NO:2) Glu-Val-Val-Arg-Glu-, X^2 is Thr, X^3 is Pro, X^4 is Met, X^5 is Ile, X^6 is Ser, X^7 is Arg, x^8 is Val, Leu, or Gly, X^9 is Gly, X^{10} is Gly, X^{11} is Asn, and X^{12} is Arg. Another aspect of this invention provides protease inhibitors wherein X^1 is (SEQ ID NO:2) Glu-Val-Val-Arg-Glu-, X^2 is Thr, X^3 is Pro, X^4 is Met, X^5 is Ile, X^6 is Ser, X^7 is Ala, x^8 is Phe, X^9 is Gly, X^{10} is Gly, X^{11} is Asn, and X^{12} is Arg. Another aspect of this invention provides protease inhibitors wherein X^1 is (SEQ ID NO:2) Glu-Val-Val-Arg-Glu-, X^2 is Thr, Val, or Ser, X^3 is Pro, X^4 is Ala or Leu, X^5 is Ile, X^6 is Tyr, X^7 His, X^8 is Phe, X^9 is Gly, X^{10} is Gly, X^{11} is Ala, and X^{12} is Arg.

Yet another aspect of this invention provides protease inhibitors wherein X^2 is Thr, and X^4 is Ala. Another aspect of this invention provides protease inhibitors wherein X^2 is Thr, and X^4 is Leu. Another aspect of this invention provides protease inhibitors wherein X^2 is Val, and X^4 is Ala. Another aspect of this invention provides protease inhibitors wherein X^2 is Ser, and X^4 is Ala. Another aspect of this invention provides protease inhibitors wherein X^2 is Val, and X^4 is Leu. Another aspect of this invention provides protease inhibitors wherein X^2 is Ser, and X^4 is Leu.

Yet another aspect of this invention provides protease inhibitors wherein X^1 is (SEQ ID NO:2) Glu-Val-Val-Arg-Glu-, X^2 is Thr, X^3 is Pro, X^4 is Leu, X^5 is Phe, X^6 is Lys, X^7 is Arg, X^8 is Phe, X^9 is Gly, X^{10} is Gly, X^{11} is Ala, and X^{12} is Arg. Another aspect of this invention provides protease inhibitors wherein X^1 is (SEQ ID NO:2) Glu-Val-Val-Arg-Glu-, X^2 is Thr, X^3 is Pro, X^4 is Leu, X^5 is Phe, X^6 is Lys, X^7 is Arg, X^8 is Phe, X^9 is Tyr, X^{10} is

Gly, X^{11} is Ala, and X^{12} is Arg. Another aspect of this invention provides protease inhibitors wherein X^1 is (SEQ ID NO:2) Glu-Val-Val-Arg-Glu-, X^2 is Thr, X^3 is Pro, X^4 is Leu, X^5 is Phe, X^6 is Lys, X^7 is Arg, X^8 is Phe, X^9 is Leu, X^{10} is Gly, X^{11} is Ala, and X^{12} is Arg.

A further aspect of this invention provides an isolated DNA molecule comprising a DNA sequence encoding a protease inhibitor of the invention. Another aspect of this invention provides an isolated DNA molecule comprising a DNA sequence encoding the protease inhibitor that further comprises an isolated DNA molecule operably linked to a regulatory sequence that controls expression of the coding sequence of the protease inhibitor in a host cell. Another aspect of this invention provides an isolated DNA molecule comprising a DNA sequence encoding the protease inhibitor operably linked to a regulatory sequence that controls expression of the coding sequence of the protease inhibitor in a host cell that further comprises a DNA sequence encoding a secretory signal peptide. That secretory signal peptide may preferably comprise the signal sequence of yeast alpha-mating factor. Another aspect of this invention provides a host cell transformed with any of the DNA molecules defined above. Such a host cell may preferably comprise *E. coli* or a yeast cell. When such a host cell is a yeast cell, the yeast cell may preferably be *Saccharomyces cerevisiae*.

Another aspect of this invention provides a method for producing a protease inhibitor of the present invention, comprising the steps of culturing a host cell as defined above and isolating and purifying said protease inhibitor.

A further aspect of this invention provides a pharmaceutical composition, comprising a protease inhibitor of the present invention together with a pharmaceutically acceptable sterile vehicle.

An additional aspect of this invention provides a method of treatment of a clinical condition associated with increased activity of one or more serine proteases,

comprising administering to a patient suffering from said clinical condition an effective amount of a pharmaceutical composition comprising a protease inhibitor of the present invention together with a pharmaceutically acceptable sterile vehicle. That method of treatment may preferably be used to treat the clinical condition of blood loss during surgery.

Yet another aspect of this invention provides a method for inhibiting the activity of serine proteases of interest in a mammal comprising administering a therapeutically effective dose of a pharmaceutical composition comprising a protease inhibitor of the present invention together with a pharmaceutically acceptable sterile vehicle.

Another aspect of this invention provides a method for inhibiting the activity of serine proteases of interest in a mammal comprising administering a therapeutically effective dose of a pharmaceutical composition comprising a protease inhibitor of the present invention together with a pharmaceutically acceptable sterile vehicle, wherein said serine proteases are selected from the group consisting of: kallikrein; chymotrypsins A and B; trypsin; elastase; subtilisin; coagulants and procoagulants, particularly those in active form, including coagulation factors such as factors VIIa, IXa, Xa, XIa, and XIIa; plasmin; thrombin; proteinase-3; enterokinase; acrosin; cathepsin; urokinase; and tissue plasminogen activator.

A further aspect of this invention relates to protease inhibitors comprising the following amino acid sequences (SEQ ID NO:3):

X^1-Val-Cys-Ser-Glu-Gln-Ala-Glu-Thr-Gly-Pro-Cys-Arg-Ala-X^2-X^3-X^4-Arg-Trp-Tyr-Phe-Asp-Val-Thr-Glu-Gly-Lys-Cys-Ala-Pro-Phe-Phe-Tyr-Gly-Gly-Cys-X^5-Gly-Asn-Arg-Asn-Asn-Phe-Asp-Thr-Glu-Glu-Tyr-Cys-Met-Ala-Val-Cys-Gly-Ser-Ala-Ile,

wherein X^1 is selected from (SEQ ID NO:2) Glu-Val-Val-Arg-Glu-, Asp, or Glu; X^2 is selected from Ala, Leu, Gly, or Met; X^3 is selected from Ile, His, Leu, Lys, Ala, or

Phe; X^4 is selected from Ser, Ile, Pro, Phe, Tyr, Trp, Asn, Leu, His, Lys, or Glu; X^5 is selected from Gly, Ala, Lys, Pro, Arg, Leu, Met, or Tyr; provided that when X^5 is Arg, X^2 is Ala or Leu; when X^5 is Tyr, X^2 is Ala or X^3 is His; and either X^3 is not Ile; or X^4 is not Ser; or X^5 is not Leu, Phe, Met, Tyr, or Asn. Another aspect of this invention provides a protease inhibitor as defined above wherein X^1 is Glu, X^2 is Met, X^3 is Ile, X^4 is Ile, and X^5 is Gly.

The invention also relates more specifically to protease inhibitors comprising the following amino acid sequences (SEQ ID NO:4):

Glu-Val-Val-Arg-Glu-Val-Cys-Ser-Glu-Gln-Ala-Glu-Thr-Gly-Pro-Cys-Arg-Ala-X^1-X^2-X^3-Arg-Trp-Tyr-Phe-Asp-Val-Thr-Glu-Gly-Lys-Cys-Ala-Pro-Phe-Phe-Tyr-Gly-Gly-Cys-X^4-Gly-Asn-Arg-Asn-Asn-Phe-Asp-Thr-Glu-Glu-Tyr-Cys-Met-Ala-Val-Cys-Gly-Ser-Ala-Ile,

wherein X^1 is selected from Ala, Leu, Gly, or Met; X^2 is selected from Ile, His, Leu, Lys, Ala, or Phe; X^3 is selected from Ser, Ile, Pro, Phe, Tyr, Trp, Asn, Leu, His, Lys, or Glu; X^4 is selected from Gly, Arg, Leu, Met, or Tyr; provided that when X^1 is Ala, X^2 is Ile, His, or Leu; when X^1 is Leu, X^2 is Ile or His; when X^1 is Leu and X^2 is Ile, X^3 is not Ser; when X^1 is Gly, X^2 is Ile; when X^4 is Arg, X^1 is Ala or Leu; when X^4 is Tyr, X^1 is Ala or X^2 is His; and either X^1 is not Met, or X^2 is not Ile, or X^3 is not Ser, or X^4 is not Gly.

A further aspect of this invention provides a protease inhibitor as defined above wherein X^1 is Met, X^3 is Ser, and X^4 is Gly. Another aspect of this invention provides a protease inhibitor wherein X^2 is selected from His, Ala, Phe, Lys, and Leu. Another aspect of this invention provides a protease inhibitor wherein X^2 is His. Another aspect of this invention provides a protease inhibitor wherein X^2 is Ala. Another aspect of this invention provides a protease inhibitor wherein X^2 is Phe. Another aspect of this invention provides a protease inhibitor wherein X^2 is Lys. Another aspect of

this invention provides a protease inhibitor wherein X^2 is Leu. Another aspect of this invention provides a protease inhibitor wherein X^1 is Met, X^2 is Ile, and X^4 is Gly.

Yet another aspect of this invention provides a protease inhibitor wherein X^3 is Ile. Another aspect of this invention provides a protease inhibitor wherein X^3 is Pro. Another aspect of this invention provides a protease inhibitor wherein X^3 is Phe. Another aspect of this invention provides a protease inhibitor wherein X^3 is Tyr. Another aspect of this invention provides a protease inhibitor wherein X^3 is Trp. Another aspect of this invention provides a protease inhibitor wherein X^3 is Asn. Another aspect of this invention provides a protease inhibitor wherein X^3 is Leu.

An additional aspect of this invention provides a protease inhibitor wherein X^3 is Lys. Another aspect of this invention provides a protease inhibitor wherein X^3 is His. Another aspect of this invention provides a protease inhibitor wherein X^3 is Glu. Another aspect of this invention provides a protease inhibitor wherein X^1 is Ala. Another aspect of this invention provides a protease inhibitor wherein X^2 is Ile. Another aspect of this invention provides a protease inhibitor wherein X^3 is Phe, and X^4 is Gly. Another aspect of this invention provides a protease inhibitor wherein X^3 is Tyr, and X^4 is Gly. Another aspect of this invention provides a protease inhibitor wherein X^3 is Trp, and X^4 is Gly.

Yet another other aspect of this invention provides a protease inhibitor wherein X^3 is Ser or Phe, and X^4 is Arg or Tyr. Another aspect of this invention provides a protease inhibitor wherein X^2 is His or Leu, X^3 is Phe, and X^4 is Gly. Another aspect of this invention provides a protease inhibitor wherein X^1 is Leu. Another aspect of this invention provides a protease inhibitor wherein X^2 is His, X^3 is Asn or Phe, and X^4 is Gly. Another aspect of this invention provides a protease inhibitor wherein X^2 is Ile, X^3 is Pro, and X^4 is Gly. Another

aspect of this invention provides a protease inhibitor wherein X^1 is Gly, X^2 is Ile, X^3 is Tyr, and X^4 is Gly. Another aspect of this invention provides a protease inhibitor wherein X^1 is Met, X^2 is His, X^3 is Ser, and X^4 is Tyr.

Additionally, another aspect of this invention relates to protease inhibitors comprising the following amino acid sequences (SEQ ID NO:5):

X^1-Val-Cys-Ser-Glu-Gln-Ala-Glu-X^2-Gly-Pro-Cys-Arg-Ala-X^3-X^4-X^5-X^6 -Arg -Trp-Tyr-Phe-Asp-Val-Thr-Glu-Gly-Lys-Cys-Ala-Pro-Phe-Phe-Tyr-Gly-Gly-Cys-X^7-Gly-Asn-Arg-Asn-Asn-Phe-Asp-Thr-Glu-Glu-Tyr-Cys-Met-Ala-Val-Cys-Gly-Ser-Ala-Ile,

wherein X^1 is selected from (SEQ ID NO:2) Glu-Val-Val-Arg-Glu-, Asp, or Glu; X^2 is selected from Thr, Val, Ile and Ser; X^3 is selected from Arg, Ala, Leu, Gly, or Met; X^4 is selected from Ile, His, Leu, Lys, Ala, or Phe; X^5 is selected from Ser, Ile, Pro, Phe, Tyr, Trp, Asn, Leu, His, Lys, or Glu; X^6 is selected from Arg, His, or Ala; and X^7 is selected from Gly, Ala, Lys, Pro, Arg, Leu, Met, or Tyr.

Another aspect of this invention provides a protease inhibitor as defined above wherein at least two amino acid residues selected from the group consisting of X^3, X^4, X^5, and X^6 differ from the residues found in the naturally occurring sequence of KPI. Another aspect of this invention provides a protease inhibitor wherein X^1 is (SEQ ID NO:2) Glu-Val-Val-Arg-Glu-, X^2 is Thr, Val, or Ser, X^3 is Ala or Leu, X^4 is Ile, X^5 is Tyr, X^6 is His and X^7 is Gly. Another aspect of this invention provides a protease inhibitor wherein X^2 is Thr, and X^3 is Ala. Another aspect of this invention provides a protease inhibitor wherein X^2 is Thr, and X^3 is Leu. Another aspect of this invention provides a protease inhibitor wherein X^2 is Val, and X^3 is Ala. Another aspect of this invention provides a protease inhibitor wherein X^2 is Ser, and X^3 is Ala. Another aspect of this invention provides a protease inhibitor wherein X^2 is Val, and X^3 is Leu. Another aspect of this invention provides a

protease inhibitor wherein X^2 is Ser, and X^3 is Leu. Another aspect of this invention provides a protease inhibitor wherein X^1 is (SEQ ID NO:2) Glu-Val-Val-Arg-Glu-, X^2 is Thr, X^3 is Leu, X^4 is Phe, X^5 is Lys, X^6 is Arg and X^7 is Gly. Another aspect of this invention provides a protease inhibitor wherein X^1 is (SEQ ID NO:2) Glu-Val-Val-Arg-Glu-, X^2 is Thr, X^3 is Leu, X^4 is Phe, X^5 is Lys, X^6 is Arg and X^7 is Tyr. Another aspect of this invention provides a protease inhibitor wherein X^1 is (SEQ ID NO:2) Glu-Val-Val-Arg-Glu-, X^2 is Thr, X^3 is Leu, X^4 is Phe, X^5 is Lys, X^6 is Arg and X^7 is Leu.

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

Brief Description of the Drawings

Figure 1 shows the strategy for the construction of plasmid pTW10:KPI.

Figure 2 shows the sequence (SEQ ID NOS:74 AND 75) of the synthetic gene for KPI (1→57) fused to the bacterial *pho*A secretory signal sequence.

Figure 3 (SEQ ID NOS 15-18) shows the strategy for construction of plasmid pKPI-61.

Figure 4 (SEQ ID NOS 76 AND 77) shows the 192 bp *Xba*I-*Hin*dIII synthetic gene fragment encoding KPI (1→57) and four amino acids from yeast alpha-mating factor.

Figure 5 (SEQ ID NOS 78 AND 79) shows the synthetic 201 bp *Xba*I-*Hin*dIII fragment encoding KPI(-4→57) in PKPI-61.

Figure 6 shows the strategy for the construction of plasmid pTW113.

Figure 7 (SEQ ID NOS 80 AND 81) shows plasmid PTW113, encoding the 445 bp synthetic gene for yeast alpha-factor-KPI(-4→57) fusion.

Figure 8 shows the amino acid sequence (SEQ ID NO:79) for KPI (-4→57).

Figure 9 (SEQ ID NOS 25 AND 26) shows the strategy for constructing plasmid pTW6165.

Figure 10 (SEQ ID NOS 82 AND 83) shows plasmid, PTW6165, encoding the 445 bp synthetic gene for alpha-factor-KPI(-4→57; M15A, S17W) fusion.

Figure 11 (SEQ ID NOS 25-42, respectfully) shows the sequences of the annealed oligonucleotide pairs used to construct plasmids PTW6165, pTW6166, pTW6175, pBG028, pTW6183, pTW6184, pTW6185, pTW6173, and pTW6174.

Figure 12 (SEQ ID NOS 84 AND 85) shows the sequence of plasmid PTW6166 encoding the fusion of yeast alpha-factor and KPI(-4→57; M15A, S17Y).

Figure 13 (SEQ ID NOS 86 AND 87) shows the sequence of plasmid PTW6175 encoding the fusion of yeast alpha-factor and KPI(-4→57; M15L, S17F).

Figure 14 shows (SEQ ID NOS 88 AND 89) the sequence of plasmid PBG028 encoding the fusion of yeast alpha-factor and KPI(-4→57; M15L, S17Y).

Figure 15 (SEQ ID NOS 90 AND 91) shows the sequence of plasmid PTW6183 encoding the fusion of yeast alpha-factor and KPI(-4→57; I16H, S17F).

Figure 16 (SEQ ID NOS 92 AND 93) shows the sequence of plasmid PTW6184 encoding the fusion of yeast alpha-factor and KPI(-4→57; I16H, S17Y).

Figure 17 (SEQ ID NOS 94 AND 95) shows the sequence of plasmid PTW6185 encoding the fusion of yeast alpha-factor and KPI(-4→57; I16H, S17W).

Figure 18 (SEQ ID NOS 96 AND 97) shows the sequence of plasmid PTW6173 encoding the fusion of yeast alpha-factor and KPI(-4→57; M15A, I16H).

Figure 19 (SEQ ID NOS 98 AND 99) shows the sequence of plasmid PTW6174 encoding the fusion of yeast alpha-factor and KPI(-4→57; M15L, I16H).

Figure 20 shows the amino acid sequence (SEQ ID NO:83) of KPI (-4→57; M15A, S17W).

Figure 21 shows the amino acid sequence (SEQ ID NO:85) of KPI (-4→57; M15A, S17Y).

Figure 22 shows the amino acid sequence (SEQ ID NO:87) of KPI (-4→57; M15L, S17F).

Figure 23 shows the amino acid sequence (SEQ ID NO:89) of KPI (-4→57; M15L, S17Y).

Figure 24 shows the amino acid sequence (SEQ ID NO:91) of KPI (-4→57; I16H, S17F).

Figure 25 shows the amino acid sequence (SEQ ID NO:93) of KPI (-4→57; I16H, S17Y).

Figure 26 shows the amino acid sequence (SEQ ID NO:95) of KPI (-4→57; I16H, S17W).

Figure 27 shows the amino acid sequence (SEQ ID NO:107) of KPI (-4→57; M15A, S17F).

Figure 28 shows the amino acid sequence (SEQ ID NO:97) of KPI (-4→57; M15A, I16H).

Figure 29 shows the amino acid sequence (SEQ ID NO:99) of KPI (-4→57; M15L, I16H).

Figure 30 (SEQ ID NOS 45-48, respectfully) shows the construction of plasmid pSP26:*Amp*:F1.

Figure 31 shows the construction of plasmid pgIII.

Figure 32 shows the construction of plasmid p*Pho*A:KPI:gIII.

Figure 33 shows the construction of plasmid pLG1.

Figure 34 (SEQ ID NOS 55 AND 56) shows the construction of plasmid pAL51.

Figure 35 shows the construction of plasmid pAL53.

Figure 36 shows the construction of plasmid PSP26:*Amp*:F1:*Pho*A:KPI:gIII.

Figure 37 shows the construction of plasmid pDW1 #14.

Figure 38 (SEQ ID NOS 100 AND 101) shows the coding region for the fusion of *pho*A-KPI (1→55)-geneIII.

Figure 39 shows the construction of plasmid PDW1 14-2.

Figure 40 shows the construction of KPI Library 16-19.

Figure 41 (SEQ ID NOS 102 AND 103) shows the expression unit encoded by the members of KPI Library 16-19.

Figure 42 (SEQ ID NOS 104 AND 105) shows the *phoA*-KPI(1→55)-geneIII region encoded by the most frequently occurring randomized KPI region.

Figure 43 shows the construction of pDD185 KPI (-4→57; M15A, S17F).

Figure 44 (SEQ ID NOS 106 AND 107) shows the sequence of alpha-factor fused to KPI (-4→57; M15A, S17F).

Figure 45 shows the inhibition constants (K_is) determined for purified KPI variants against the selected serine proteases kallikrein, factor Xa, and factor XIIa.

Figure 46 (SEQ ID NOS 108-228, respectfully) shows the inhibition constants (K_is) determined for KPI variants against kallikrein, plasmin, and factors Xa, XIa, and XIIa.

Figure 47 shows the post-surgical blood loss in pigs in the presence (KPI) and absence (NS) of KPI 185-1 (M15A, S17F).

Figure 48 shows the post-surgical hemoglobin loss in pigs in the presence (KPI) and absence (NS) of KPI 185-1 (M15A, S17F).

Figure 49 shows the oxygen tension in the presence and absence of KPI, before CPB, immediately after CPB, and at 60 and 180 minutes after the end of CPB.

Figure 50 summarizes the results shown in Figures 47-49.

Detailed Description

The present invention provides peptides that can bind to and preferably inhibit the activity of serine proteases. These inhibitory peptides can also provide a means of ameliorating, treating or preventing clinical conditions associated with increased activity of serine proteases. The novel peptides of the present invention preferably exhibit a more potent and specific (i.e., greater) inhibitory effect toward serine proteases of interest than known serine protease inhibitors. Examples of such proteases include: kallikrein; chymotrypsins A and B; trypsin; elastase; subtilisin; coagulants and procoagulants, particularly those in active form, including coagulation factors such as factors VIIa, IXa, Xa, XIa, and XIIa; plasmin; thrombin; proteinase-3; enterokinase; acrosin; cathepsin; urokinase; and tissue plasminogen activator.

Peptides of the present invention may be used to reduce the tissue damage caused by activation of the proteases of the contact pathway of the blood during surgical procedures such as cardiopulmonary bypass (CPB). Inhibition of contact pathway proteases reduces the "whole body inflammatory response" that can accompany contact pathway activation, and that can lead to tissue damage, and possibly death. The peptides of the present invention may also be used in conjunction with surgical procedures to reduce activated serine protease-associated perioperative and postoperative blood loss. For instance, perioperative blood loss of this type may be particularly severe during CPB surgery. Pharmaceutical compositions comprising the peptides of the present invention may be used in conjunction with surgery such as CPB; administration of such compositions may occur preoperatively, perioperatively or postoperatively. Examples of other clinical conditions associated with increased serine protease activity for which the peptides of the present invention may be used include: CPB-induced inflammatory response; post-CPB pulmonary injury; pancreatitis; allergy-induced protease release; deep vein

thrombosis; thrombocytopenia; rheumatoid arthritis; adult respiratory distress syndrome; chronic inflammatory bowel disease; psoriasis; hyperfibrinolytic hemorrhage; organ preservation; wound healing; and myocardial infarction. Other examples of preferable uses of the peptides of the present invention are described in U.S. Patent No. 5,187,153.

The invention is based upon the novel substitution of amino acid residues in the peptide corresponding to the naturally occurring KPI protease inhibitor domain of human amyloid β-amyloid precursor protein (APPI). These substitutions produce peptides that can bind to serine proteases and preferably exhibit an inhibition of the activity of serine proteases. The peptides also preferably exhibit a more potent and specific serine protease inhibition than known serine protease inhibitors. In accordance with the invention, peptides are provided that may exhibit a more potent and specific inhibition of one or more serine proteases of interest, e.g., kallikrein, plasmin and factors Xa, XIa, XIIa, and XIIa.

The present invention also includes pharmaceutical compositions comprising an effective amount of at least one of the peptides of the invention, in combination with a pharmaceutically acceptable sterile vehicle, as described in REMINGTON'S PHARMACEUTICAL SCIENCES: DRUG RECEPTORS AND RECEPTOR THEORY, (18th ed.), Mack Publishing Co., Easton, PA (1990).

A. Selection of sequences of KPI variants

The sequence of KPI is shown in Table 1. Table 2 shows a comparison of this sequence with that of aprotinin, with which it shares about 45% sequence identity. The numbering convention for KPI shown in Table 1 and used hereinafter designates the first glutamic acid residue of KPI as residue 1. This corresponds to residue number 3 using the standard numbering convention for aprotinin.

The crystal structure for KPI complexed with trypsin has been determined. See Perona *et al.*, *J. Mol. Biol.* 230:919 (1993). The three-dimensional structure reveals two binding loops within KPI that contact the protease. The first loop extends from residue Thr^{9} to Ile^{16}, and the second loop extends from residue Phe^{32} to Gly^{37}. The two protease binding loops are joined through the disulfide bridge extending from Cys^{12} to Cys^{36}. KPI contains two other disulfide bridges, between Cys^{3} and Cys^{53}, and between Cys^{28} to Cys^{49}.

This structure was used as a guide to inform our strategy for making the amino acid residue substitutions that will be most likely to affect the protease inhibitory properties of KPI. Our examination of the structure indicated that certain amino acid residues, including residues 9, 11, 13-18, 32, and 37-40, appear to be of particular significance in determining the protease binding properties of the KPI peptide. In a preferred embodiment of the invention two or more of those KPI peptide residues are substituted; such substitutions preferably occurring among residues 9, 11, 13-18, 32, and 37-40. In particular, we found that those substituted peptides, including peptides comprising substitutions of at least two of the four residues at positions 15-18, may exhibit more potent and specific serine protease inhibition toward selected serine proteases of interest than exhibited by the natural KPI peptide domain. Such substituted peptides may further comprise one or more additional substitutions at residues 9, 11, 13, 14, 32 and 37-40; in particular, such peptides may further comprise a substitution at positions 9 or 37. In particular, the peptides of the present invention preferably exhibit a greater potency and specificity for inhibiting one or more serine proteases of interest (e.g., kallikrein, plasmin and factors VIIa, IXa, Xa, XIa, and XIIa) than the potency and specificity exhibited by native KPI or other known serine protease inhibitors. That greater potency and specificity may be manifested by the peptides of the present invention by exhibiting

binding constants for serine proteases of interest that are less than the binding constants exhibited by native KPI, or other known serine protease inhibitors, for such proteases.

By way of example, and as set forth in greater detail below, the serine protease inhibitory properties of peptides of the present invention were measured for the serine proteases of interest — kallikrein, plasmin and factors Xa, XIa, and XIIa. Methodologies for measuring the inhibitory properties of the KPI variants of the present invention are known to those skilled in the art, e.g., by determining the inhibition constants of the variants toward serine proteases of interest, as described in Example 4, *infra*. Such studies measure the ability of the novel peptides of the present invention to bind to one or more serine proteases of interest and to preferably exhibit a greater potency and specificity for inhibiting one or more serine protease of interest than known serine protease inhibitors such as native KPI.

The ability of the peptides of the present invention to bind one or more serine proteases of interest, particularly the ability of the peptides to exhibit such greater potency and specificity toward serine proteases of interest, manifest the clinical and therapeutic applications of such peptides. The clinical and therapeutic efficacy of the peptides of the present invention can be assayed by *in vitro* and *in vivo* methodologies known to those skilled in the art, e.g., as described in Example 5, *infra*.

Table 1 (SEQ ID NO:6): SEQUENCE OF KPI:

```
  1                  10                  20                  30
V R E V C S E Q A E T G P C R A M I S R W Y F D V T E G K C A P

                    40                  50
F F Y G G C G G N R N N F D T E E Y C M A V C G S A I
```

Table 2 (SEQ ID NOS 6 AND 7): COMPARISON OF KPI AND APROTININ SEQUENCES:

```
          1         10        20 30       40        50
KPI:  VREVCSEQAETGPCRAMISRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI
          | |   |||| | | |       | |  | ||||   ||||   | ||  || |
BPTI: RPDFCLEPPYTGPCKARIIRYFYNAKAGLCQTFVYGGCRAKRNNFKSAEDCMRTCGGA
          1       10        20        30       40        50
```

B. Methods of producing KPI variants

The peptides of the present invention can be created by synthetic techniques or recombinant techniques which employ genomic or cDNA cloning methods.

1. Production by chemical synthesis

Peptides of the present invention can be routinely synthesized using solid phase or solution phase peptide synthesis. Methods of preparing relatively short peptides such as KPI by chemical synthesis are well known in the art. KPI variants could, for example be produced by solid-phase peptide synthesis techniques using commercially available equipment and reagents such as those available from Milligen (Bedford, MA) or Applied Biosystems-Perkin Elmer (Foster City, CA). Alternatively, segments of KPI variants could be prepared by solid-phase synthesis and linked together using segment condensation methods such as those described by Dawson *et al.*, *Science* 266:776 (1994). During chemical synthesis of the KPI variants, substitution of any amino acid is achieved simply by replacement of the residue that is to be substituted with a different amino acid monomer.

2. Production by recombinant DNA technology

(a) Preparation of genes encoding KPI variants

In a preferred embodiment of the invention, KPI variants are produced by recombinant DNA technology. This requires the preparation of genes encoding each KPI variant that is to be made. Suitable genes can be constructed by oligonucleotide synthesis using commercially available equipment, such as that provided by Milligen and Applied Biosystems, *supra*. The genes can be prepared by synthesizing the entire coding and non-coding strands, followed by annealing the two strands. Alternatively, the genes can be prepared by ligation of smaller synthetic oligonucleotides by methods well known in the art. Genes encoding KPI variants are produced by

varying the nucleotides introduced at any step of the synthesis to change the amino acid sequence encoded by the gene.

Preferably, however, KPI variants are made by site-directed mutagenesis of a gene encoding KPI. Methods of site-directed mutagenesis are well known in the art. See, for example, Ausubel *et al.*, (eds.) CURRENT PROTOCOLS IN MOLECULAR BIOLOGY (Wiley Interscience, 1987); PROTEIN ENGINEERING (Oxender & Fox eds., A. Liss, Inc. 1987). These methods require the availability of a gene encoding KPI or a variant thereof, which can then be mutagenized by known methods to produce the desired KPI variants. In addition, linker-scanning and polymerase chain reaction ("PCR") mediated techniques can be used for purposes of mutagenesis. See PCR TECHNOLOGY (Erlich ed., Stockton Press 1989); CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, vols. 1 & 2, *loc. cit.*

A gene encoding KPI can be obtained by cloning the naturally occurring gene, as described for example in U.S. Patents Nos. 5,223,482 and 5,187,153, which are hereby incorporated by reference in their entireties. In particular, see columns 6-9 of U.S. Patent No. 5,187,153. See also PCT Application No. 93/09233. In a preferred embodiment of the invention a synthetic gene encoding KPI is produced by chemical synthesis, as described above. The gene may encode the 57-amino acid KPI domain shown in Table 1, or it may also encode additional N-terminal amino acids from the APPI protein sequence, such as the four amino acid sequence (SEQ ID NO:8) (Glu-Val-Val-Arg, designated residues -4 to -1) immediately preceding the KPI domain in APPI.

Production of the gene by synthesis allows the codon usage of the KPI gene to be altered to introduce convenient restriction endonuclease recognition sites, without altering the sequence of the encoded peptide. In a preferred embodiment of the invention, the synthetic KPI gene contains restriction endonuclease recognition sites that facilitate excision of DNA cassettes from the KPI gene. These cassettes can be replaced with small

synthetic oligonucleotides encoding the desired changes in the KPI peptide sequence. See Ausubel, *supra*.

This method also allows the production of genes encoding KPI as a fusion peptide with one or more additional peptide or protein sequences. The DNA encoding these additional sequences is arranged in-frame with the sequence encoding KPI such that, upon translation of the gene, a fusion protein of KPI and the additional peptide or protein sequence is produced. Methods of making such fusion proteins are well known in the art. Examples of additional peptide sequences that can be encoded in the genes are secretory signal peptide sequences, such as bacterial leader sequences, for example *ompA* and *phoA*, that direct secretion of proteins to the bacterial periplasmic space. In a preferred embodiment of the invention, the additional peptide sequence is a yeast secretory signal sequence, such as α-mating factor, that directs secretion of the peptide when produced in yeast.

Additional genetic regulatory sequences can also be introduced into the synthetic gene that are operably linked to the coding sequence of the gene, thereby allowing synthesis of the protein encoded by the gene when the gene is introduced into a host cell. Examples of regulatory genetic sequences that can be introduced are: promoter and enhancer sequences and transcriptional and translational control sequences. Other regulatory sequences are well known in the art. See Ausubel *et al.*, *supra*, and Sambrook *et al.*, *supra*.

Sequences encoding other fusion proteins and genetic elements are well known to those of skill in the art. In a preferred embodiment of the invention, the KPI sequence is prepared by ligating together synthetic oligonucleotides to produce a gene encoding an in-frame fusion protein of yeast α-mating factor with either KPI (1→57) or KPI (-4→57).

The gene constructs prepared as described above are conveniently manipulated in host cells using methods of manipulating recombinant DNA techniques that are well

known in the art. See, for example Sambrook *et al.*, MOLECULAR CLONING: A LABORATORY MANUAL, Second Edition, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY 1989), and Ausubel, *supra*. In a preferred embodiment of the invention the host cell used for manipulating the KPI constructs is *E. coli*. For example, the construct can be ligated into a cloning vector and propagated in *E. coli* by methods that are well known in the art. Suitable cloning vectors are described in Sambrook, *supra*, or are commercially available from suppliers such as Promega (Madison, WI), Stratagene (San Diego, CA) and Life Technologies (Gaithersburg, MD).

Once a gene construct encoding KPI has been obtained, genes encoding KPI variants are obtained by manipulating the coding sequence of the construct by standard methods of site-directed mutagenesis, such as excision and replacement of small DNA cassettes, as described *supra*. See Ausubel, *supra*, and Sinha *et al.*, *supra*. See also U.S. Patent 5,373,090, which is herein incorporated by reference in its entirety. See particularly, columns 4-12 of U.S. Patent 5,272,090. These genes are then used to produce the KPI variant peptides as described below.

Alternatively, KPI variants can be produced using phage display methods. See, for example, Dennis *et al.* *supra*, which is hereby incorporated by reference in its entirety. See also U.S. Patent Nos. 5,223,409 and 5,403,484, which are hereby also incorporated by reference in their entireties. In these methods, libraries of genes encoding variants of KPI are fused in-frame to genes encoding surface proteins of filamentous phage, and the resulting peptides are expressed (displayed) on the surface of the phage. The phage are then screened for the ability to bind, under appropriate conditions, to serine proteases of interest immobilized on a solid support. Large libraries of phage can be used, allowing simultaneous screening of the binding properties of a large number of KPI variants. Phage that have desirable binding properties are isolated and the sequences of the genes encoding the corresponding KPI

variants is determined. These genes are then used to produce the KPI variant peptides as described below.

(b) Expression of KPI variant peptides

Once genes encoding KPI variants have been prepared, they are inserted into an expression vector and used to produce the recombinant peptide. Suitable expression vectors and corresponding methods of expressing recombinant proteins and peptides are well known in the art. Methods of expressing KPI peptides are described in U.S. Patent 5,187,153, columns 9-11, U.S. Patent 5,223,482, columns 9-11, and PCT application 93/09233, pp. 49-67. See also Ausubel *et al.*, *supra*, and Sambrook *et al.*, *supra*. The gene can be expressed in any number of different recombinant DNA expression systems to generate large amounts of the KPI variant, which can then be purified and tested for its ability to bind to and inhibit serine proteases of interest.

Examples of expression systems known to the skilled practitioner in the art include bacteria such as *E. coli*, yeast such as *Saccharomyces cerevisiae* and *Pichia pastoris*, baculovirus, and mammalian expression systems such as in Cos or CHO cells. In a preferred embodiment, KPI variants are expressed in *S. cerevisiae*. In another preferred embodiment the KPI variants are cloned into expression vectors to produce a chimeric gene encoding a fusion protein of the KPI variant with yeast α-mating factor. The mating factor acts as a signal sequence to direct secretion of the fusion protein from the yeast cell, and is then cleaved from the fusion protein by a membrane-bound protease during the secretion process. The expression vector is transformed into *S. cerevisiae*, the transformed yeast cells are cultured by standard methods, and the KPI variant is purified from the yeast growth medium.

Recombinant bacterial cells expressing the peptides of the present invention, for example, *E. coli*, are grown in any of a number of suitable media, for example LB, and the expression of the recombinant antigen induced by

adding IPTG to the media or switching incubation to a higher temperature. After culturing the bacteria for a further period of between 2 and 24 hours, the cells are collected by centrifugation and washed to remove residual media. The bacterial cells are then lysed, for example, by disruption in a cell homogenizer and centrifuged to separate dense inclusion bodies and cell membranes from the soluble cell components. This centrifugation can be performed under conditions whereby dense inclusion bodies are selectively enriched by incorporation of sugars such as sucrose into the buffer and centrifugation at a selective speed. If the recombinant peptide is expressed in inclusion bodies, as is the case in many instances, these can be washed in any of several solutions to assist in the removal of any contaminating host proteins, then solubilized in solutions containing high concentrations of urea (e.g., 8M) or chaotropic agents such as guanidine hydrochloride in the presence of reducing agents such as β-mercaptoethanol or DTT (dithiothreitol).

At this stage it may be advantageous to incubate the peptides of the present invention for several hours under conditions suitable for the peptides to undergo a refolding process into a conformation which more closely resembles that of native KPI. Such conditions generally include low protein concentrations less than 500 μg/ml, low levels of reducing agent, concentrations of urea less than 2M and often the presence of reagents such as a mixture of reduced and oxidized glutathione which facilitate the interchange of disulphide bonds within the protein molecule. The refolding process can be monitored, for example, by SDS-PAGE or with antibodies which are specific for the native molecule (which can be obtained from animals vaccinated with the native molecule isolated from parasites). Following refolding, the peptide can then be purified further and separated from the refolding mixture by chromatography on any of several supports including ion exchange resins, gel permeation resins or on a variety of affinity columns.

Purification of KPI variants can be achieved by standard methods of protein purification, e.g., using various chromatographic methods including high performance liquid chromatography and adsorption chromatography. The purity and the quality of the peptides can be confirmed by amino acid analyses, molecular weight determination, sequence determination and mass spectrometry. See, for example, PROTEIN PURIFICATION METHODS — A PRACTICAL APPROACH, Harris *et al.*, eds. (IRL Press, Oxford, 1989). In a preferred embodiment, the yeast cells are removed from the growth medium by filtration or centrifugation, and the KPI variant is purified by affinity chromatography on a column of trypsin-agarose, followed by reversed-phase HPLC.

C. Measurement of protease inhibitory properties of KPI variants

Once KPI variants have been purified, they are tested for their ability to bind to and inhibit serine proteases of interest *in vitro*. The peptides of the present invention preferably exhibit a more potent and specific inhibition of serine proteases of interest than known serine protease inhibitors, such as the natural KPI peptide domain. Such binding and inhibition can be assayed for by determining the inhibition constants for the peptides of the present invention toward serine proteases of interest and comparing those constants with constants determined for known serine protease inhibitors, e.g., the native KPI domain, toward those proteases. Methods for determining inhibition constants of protease inhibitors are well known in the art. See Fersht, ENZYME STRUCTURE AND MECHANISM, 2nd ed., W.H. Freeman and Co., New York, (1985).

In a preferred embodiment the inhibition experiments are carried out using a chromogenic synthetic protease substrate, as described, for example, in Bender *et al.*, *J. Amer. Chem. Soc.* 88:5890 (1966). Measurements taken by this method can be used to calculate inhibition

constants (K_i values) of the peptides of the present invention toward serine proteases of interest. See Bieth in BAYER-SYMPOSIUM V "PROTEINASE INHIBITORS", Fritz *et al.*, eds., pp. 463-69, Springer-Verlag, Berlin, Heidelberg, New York, (1974). KPI variants that exhibit potent and specific inhibition of one or more serine proteases of interest may subsequently be tested *in vivo*. *In vitro* testing, however, is not a prerequisite for *in vivo* studies of the peptides of the present invention.

D. Testing of KPI variants *in vivo*

The peptides of the present invention may be tested, alone or in combination, for their therapeutic efficacy by various *in vivo* methodologies known to those skilled in the art, e.g., the ability of KPI variants to reduce postoperative bleeding can be tested in standard animal models. For example, cardiopulmonary bypass surgery can be carried out on animals such as pigs in the presence of KPI variants, or in control animals where the KPI variant is not used. The use of pigs as a model for studying the clinical effects associated with CPB has previously been described. See Redmond *et al.*, *Ann. Thorac. Surg.* 56:474 (1993).

The KPI variant is supplied to the animals in a pharmaceutical sterile vehicle by methods known in the art, for example by continuous intravenous infusion. Chest tubes can be used to collect shed blood for a defined period of time. The shed blood, together with the residual intrathoracic blood found after sacrifice of the animal can be used to calculate hemoglobin (Hgb) loss. The postoperative blood and Hgb loss is then compared between the test and control animals to determine the effect of the KPI variants.

E. Therapeutic use of KPI variants

KPI variants of the present invention found to exhibit therapeutic efficacy (e.g., reduction of blood loss following surgery in animal models) may preferably be used and administered, alone or in combination or as

a fusion protein, in a manner analogous to that currently used for aprotinin or other known serine protease inhibitors. See Butler *et al.*, *supra*. Peptides of the present invention generally may be administered in the manner that natural peptides are administered. A therapeutically effective dose of the peptides of the present invention preferably affects the activity of the serine proteases of interest such that the clinical condition may be treated, ameliorated or prevented. Therapeutically effective dosages of the peptides of the present invention can be determined by those skilled in the art, e.g., through *in vivo* or *in vitro* models. Generally, the peptides of the present invention may be administered in total amounts of approximately 0.01 to approximately 500, specifically 0.1 to 100 mg/kg body weight, if desired in the form of one or more administrations, to achieve therapeutic effect. It may, however, be necessary to deviate from such administration amounts, in particular depending on the nature and body weight of the individual to be treated, the nature of the medical condition to be treated, the type of preparation and the administration of the peptide, and the time interval over which such administration occurs. Thus, it may in some cases be sufficient to use less than the above amount of the peptides of the present invention, while in other cases the above amount is preferably exceeded. The optimal dose required in each case and the type of administration of the peptides of the present invention can be determined by one skilled in the art in view of the circumstances surrounding such administration. Such peptides can be administered by intravenous injections, *in situ* injections, local applications, inhalation, oral administration using coated polymers, dermal patches or other appropriate means. Compositions comprising peptides of the present invention are advantageously administered in the form of injectable compositions. Such peptides may be preferably administered to patients via continuous intravenous infusion, but can also be administered by single or

multiple injections. A typical composition for such purpose comprises a pharmaceutically acceptable carrier. Pharmaceutically acceptable carriers include aqueous solutions, non-toxic excipients, including salts, preservatives, buffers and the like, as described in REMINGTON'S PHARMACEUTICAL SCIENCES, pp. 1405-12 and 1461-87 (1975) and THE NATIONAL FORMULARY XIV., 14th Ed. Washington: American Pharmaceutical Association (1975). Aqueous carriers include water, alcoholic/aqueous solutions, saline solutions, parenteral vehicles such as sodium chloride, Ringer's dextrose, etc. Intravenous vehicles include fluid and nutrient replenishers. Preservatives include antimicrobials, anti-oxidants, chelating agents and inert gases. The pH and exact concentration of the various components of the composition are adjusted according to routine skills in the art. See GOODMAN AND GILMAN'S THE PHARMACOLOGICAL BASIS FOR THERAPEUTICS (7th ed.). The peptides of the present invention may be present in such pharmaceutical preparations in a concentration of approximately 0.1 to 99.5% by weight, specifically 0.5 to 95% by weight, relative to the total mixture. Such pharmaceutical preparations may also comprise other pharmaceutically active substances in addition to the peptides of the present invention. Other methods of delivering the peptides to patients will be readily apparent to the skilled artisan.

Examples of mammalian serine proteases that may exhibit inhibition by the peptides of the present invention include: kallikrein; chymotrypsins A and B; trypsin; elastase; subtilisin; coagulants and procoagulants, particularly those in active form, including coagulation factors such as thrombin and factors VIIa, IXa, Xa, XIa, and XIIa; plasmin; proteinase-3; enterokinase; acrosin; cathepsin; urokinase; and tissue plasminogen activator. Examples of conditions associated with increased serine protease activity include: CPB-induced inflammatory response; post-CPB pulmonary injury; pancreatitis; allergy-induced

protease release; deep vein thrombosis; thrombocytopenia; rheumatoid arthritis; adult respiratory distress syndrome; chronic inflammatory bowel disease; psoriasis; hyperfibrinolytic hemorrhage; organ preservation; wound healing; and myocardial infarction. Other examples of the use of the peptides of the present invention are described in U.S. Patent No. 5,187,153.

The inhibitors of the present invention may also be used for inhibition of serine protease activity *in vitro*, for example during the preparation of cellular extracts to prevent degradation of cellular proteins. For this purpose the inhibitors of the present invention may preferably be used in a manner analogous to the way that aprotinin, or other known serine protease inhibitors, are used. The use of aprotinin as a protease inhibitor for preparation of cellular extracts is well known in the art, and aprotinin is sold commercially for this purpose.

The present invention, thus generally described, will be understood more readily by reference to the following examples, which are provided by way of illustration and are not intended to be limiting of the present invention.

EXAMPLES

Example 1. Expression of wild-type KPI (-4→57)

A. Construction of PTW10:KPI

Plasmid PTW10:KPI is a bacterial expression vector encoding the 57 amino acid form of KPI fused to the bacterial *pho*A signal sequence. The strategy for the construction of PTW10:KPI is shown in Figure 1.

Plasmid pcDNAII (Invitrogen, San Diego, CA) was digested with *Pvu*II and the larger of the two resulting *Pvu*II fragments (3013 bp) was isolated. Bacterial expression plasmid pSP26 was digested with *Mlu*I and *Rsr*II, and the 409 bp *Mlu*I-*Rsr*II fragment containing the pTrp promoter element and transcription termination signals was isolated by electrophoresis in a 3% NuSieve Agarose gel (FMC Corp., Rockland, ME). Plasmid pSP26, containing a heparin-binding EGF-like growth factor (HB-EGF) insert between the *Nde*I and *Hin*dIII sites, is

described as pNA28 in Thompson *et al.*, *J. Biol. Chem.* 269:2541 (1994). Plasmid pSP26 was deposited in host *E. coli* W3110, pSP26 with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland, 20852, USA under the conditions specified by the Budapest Treaty on the International Recognition of the Deposit of Microorganisms (Budapest Treaty). Host *E. coli* W3110, pSP26 was deposited on 3 May 1995 and given Accession No. 69800. Availability of the deposited plasmid is not to be construed as a license to practice the invention in contravention of the rights granted under the authority of any government in accordance with its patent laws.

The ends of the *Mlu*I-*Rsr*II fragment were blunted using DNA polymerase Klenow fragment by standard techniques. The blunted fragment of pSP26 was then ligated into the large *Pvu*II fragment of plasmid pCDNAII, and the ligation mixture was used to transform *E. coli* strain MC1061. Ampicillin-resistant colonies were selected and used to isolate plasmid pTW10 by standard techniques.

A synthetic gene was constructed encoding the bacterial *pho*A secretory signal sequence fused to the amino terminus of KPI(1→57). The synthetic gene contains cohesive ends for *Nde*I and *Hind*III, and also incorporates restriction endonuclease recognition sites for *Age*I, *Rsr*II, *Aat*II and *Bam*HI, as shown in Figure 2. The synthetic *pho*A-KPI gene was constructed from 6 oligonucleotides of the following sequences (shown 5'→3'):

6167 (SEQ ID NO:9):
TATGAAACAAAGCACTATTGCACTGGCACTCTTACCGTTACTGTTTACCC
CTGTGACAAAAGCCGAGGTGTGCTCTGAA

6169 (SEQ ID NO:10):
CTCGGCTTTTGTCACAGGGGTAAACAGTAACGGTAAGAGTGCCAGTGCAA
TAGTGCTTTGTTTCATA

6165 (SEQ ID NO:11):
CAAGCTGAGACCGGTCCGTGCCGTGCAATGATCTCCCGCTGGTACTTTGA
CGTCACTGAAGGTAAGTGCGCTCCATTCTTT

6166 (SEQ ID NO:12):
GCACTTACCTTCAGTGACGTCAAAGTACCAGCGGGAGATCATTGCACGGC
ACGGACCGGTCTCAGCTTGTTCAGAGCACAC

6168 (SEQ ID NO:13):
TACGGCGGTTGCGGCGGCAACCGTAACAACTTTGACACTGAAGAGTACTG
CATGGCAGTGTGCGGATCCGCTATTTAAGCT

6164 (SEQ ID NO:14):
AGCTTAAATAGCGGATCCGCACACTGCCATGCAGTACTCTTCAGTGTCAA
AGTTGTTACGGTTGCCGCCGCAACCGCCGTAAAAGAATGGAGC

The oligonucleotides were phosphorylated and annealed in pairs: 6167 + 6169, 6165 + 6166, 6168 + 6164. In 20 μl T4 DNA Ligase Buffer (New England Biolabs, Beverley, MA), 1 μg of each oligonucleotide pair was incubated with 10 U T4 Polynucleotide Kinase (New England Biolabs) for 1 h at 37°C, then heated to 95°C for 1 minute, and slow-cooled to room temperature to allow annealing. All three annealed oligo pairs were then mixed for ligation to one another in a total volume of 100 μl T4 DNA Ligase Buffer, and incubated with 400 U T4 DNA Ligase (New England Biolabs) overnight at 15°C. The ligation mixture was extracted with an equal volume of phenol:$CHCl_3$ (1:1), ethanol-precipitated, resuspended in 50 μl Restriction Endonuclease Buffer #4 (New England Biolabs) and digested with *Nde*I and *Hin*dIII. The annealed, ligated and digested oligos were then subjected to electrophoresis in a 3% NuSieve Agarose gel, and the 240 bp *Nde*I-*Hin*dIII fragment was excised. This gel-purified synthetic gene was ligated into plasmid pTW10 which had previously been digested with *Nde*I and *Hin*dIII, and the ligation mixture was used to transform *E. coli* strain MC1061. Ampicillin-resistant colonies were selected and used to prepare plasmid pTW10:KPI. This plasmid contains the *pho*A-KPI(1→57) fusion protein inserted between the pTrp promoter element and the transcription termination signals.

B. Construction of pKPI-61

The strategy for constructing pKPI-61 is shown in Figure 3. Plasmid pTW10:KPI was digested with *Age*I and

*Hin*dIII; the resulting 152 bp *Age*I-*Hin*dIII fragment containing a portion of the KPI synthetic gene was isolated by preparative gel electrophoresis. An oligonucleotide pair (129 + 130) encoding the 9 amino-terminal residues of KPI(1→57) and 4 amino acids of yeast α-mating factor was phosphorylated and annealed as described above.

129 (SEQ ID NO:15): CTAGATAAAAGAGAGGTGTGCTCTGAACAAGCTGAGA
130 (SEQ ID NO:16): CCGGTCTCAGCTTGTTCAGAGCACACCTCTCTTTTAT

The annealed oligonucleotides were then ligated to the *Age*I-*Hin*dIII fragment of the KPI (1→57) synthetic gene. The resulting 192 bp *Xba*I-*Hin*dIII synthetic gene (shown in Figure 4) was purified by preparative gel electrophoresis, and ligated into plasmid pUC19 which had previously been digested with *Xba*I and *Hin*dIII. The ligation products were used to transform *E. coli* strain MC1061. Ampicillin-resistant colonies were picked and used to prepare plasmid PKPI-57 by standard methods. To create a synthetic gene encoding KPI(-4→57), PKPI-57 was digested with *Xba*I and *Age*I and the smaller fragment replaced with annealed oligos 234 + 235, which encode 4 amino acid residues of yeast α-mating factor fused a 4 amino acid residue amino-terminal extension of KPI(1→57).

234 (SEQ ID NO:17):
CTAGATAAAAGAGAGGTTGTTAGAGAGGTGTGCTCTGAACAAGCTGAGA

235 (SEQ ID NO:18):
CCGGTCTCAGCTTGTTCAGAGCACACCTCTCTAACAACCTCTCTTTTAT

The 4 extra amino acids are encoded in the amyloid β-protein precursor/protease nexin-2 (APPI) which contains the KPI domain. The synthetic 201 bp *Xba*I-*Hin*dIII fragment encoding KPI(-4→57) in pKPI-61 is shown in Figure 5.

C. Assembly of pTW113

The strategy for the construction of PTW113 is shown in Figure 6. Plasmid pSP35 was constructed from yeast expression plasmid pYES2 (Invitrogen, San Diego, CA) as follows. A 267 bp *Pvu*II-*Xba*I fragment was generated by PCR from yeast α-mating factor DNA using oligos 6274 and 6273:

6274 (SEQ ID NO:19): GGGGGCAGCTGTATAAACGATTAAAA

6273 (SEQ ID NO:20): GGGGGTCTAGAGATACCCCTTCTTCTTTAG

This PCR fragment, encoding an 82 amino acid portion of yeast α-mating factor, including the secretory signal peptide and pro-region, was inserted into pYES2 that had been previously digested with *Pvu*II and *Xba*I. The resulting plasmid is denoted pSP34.

Two oligonucleotide pairs, 6294 + 6292 were then ligated to 6290 + 6291, and the resulting 135 bp fragment was purified by gel electrophoresis.

6294 (SEQ ID NO:21):
CTAGATAAAAGAGAGGCTGAGGCTCACGCTGAAGGTACTTTCACTTC

6290 (SEQ ID NO:22): TGACGTCTCTTCTTACTTGGAAGGTCAAGC
TGCTAAGGAATTCATCGCTTGGTTGGTCAA
AGGTAGAGGTTAAGCTTA

6291 (SEQ ID NO:23): CTAGTAAGCTTAACCTCTACCTTTGACCAA
CCAAGCGATGAATTCCTTAGCA

6292 (SEQ ID NO:24): GCTTGACCTTCCAAGTAAGAAGAGACGTCA
GAAGTGAAAGTACCTTCAGCGTGAGCCTCA
GCCTCTCTTTTAT

The resulting synthetic fragment was ligated into the *Xba*I site of pSP34, resulting in plasmid pSP35. pSP35 was digested with *Xba*I and *Hind*III to remove the insert, and ligated with the 201 bp *Xba*I-*Hind*III fragment of pKPI-61, encoding KPI(-4→57). The resulting plasmid pTW113, encodes the 445 bp synthetic gene for the α-factor-KPI(-4→57) fusion. See Figure 7.

D. Transformation of yeast with pTW113

Saccharomyces cerevisiae strain ABL115 was transformed with plasmid pTW113 by electroporation by the method of Becker *et al.*, *Methods Enzymol.* 194:182 (1991). An overnight culture of yeast strain ABL115 was used to inoculate 200 ml YPD medium. The inoculated culture was grown with vigorous shaking at 30°C to an OD_{600} of 1.3-1.5, at which time the cells were harvested by centrifugation at 5000 rpm for 5 minutes. The cell pellet was resuspended in 200 ml ice-cold water, respun, resuspended in 100 ml ice-cold water, then pelleted again. The washed cell pellet was resuspended in 10 ml ice-cold 1M sorbitol, recentrifuged, then resuspended in a final volume of 0.2 ml ice-cold 1M sorbitol. A 40 µl aliquot of cells was placed into the chamber of a cold 0.2 cm electroporation cuvette (Invitrogen), along with 100 ng plasmid DNA for pTW113. The cuvette was placed into an Invitrogen Electroporator II and pulsed at 1500 V, 25 µF, 100 Ω. Electroporated cells were diluted with 0.5 ml 1M sorbitol, and 0.25 ml was spread on an SD agar plate containing 1M sorbitol. After 3 days' growth at 30°C, individual colonies were streaked on SD + CAA agar plates.

E. Induction of pTW113/ABL115, purification of KPI(-4→57)

Yeast cultures were grown in a rich broth and the galactose promoter of the KPI expression vector induced with the addition of galactose as described by Sherman, *Methods Enzymol.* 194:3 (1991). A single well-isolated colony of pTW113/ABL115 was used to inoculate a 10 ml overnight culture in Yeast Batch Medium. The next day, 1L Yeast Batch Medium which had been made 0.2% glucose was inoculated to an OD_{600} of 0.1 with the overnight culture. Following 24 hours at 30°C with vigorous shaking, the 1L culture was induced by the addition of 20 ml Yeast Galactose Feed Medium. Following induction, the culture was fed every 12 hours with the addition of 20 ml Yeast Galactose Feed Medium. At 48 hours afterinduction,

the yeast broth was harvested by centrifugation, then adjusted to pH 7.0 with 2M Tris, pH 10. The broth was subjected to trypsin-Sepharose affinity chromatography, and bound KPI(-4→57) was eluted with 20mM Tris pH 2.5. See Schilling *et al.*, *Gene* 98:225 (1991). Final purification of KPI(-4→57) was accomplished by HPLC chromatography on a semi-prep Vydac C4 column in a gradient of 20% to 35% acetonitrile. The sample was dried and resuspended in PBS at 1-2 mg/ml. The amino acid sequence of KPI(-4→57) is shown in Figure 8.

Example 2. Recombinant Expression of site-directed KPI(-4→57) variants

Expression vectors for the production of specific variants of KPI(-4→57) were all constructed using the pTW113 backbone as a starting point. For each KPI variant, an expression construct was created by replacing the 40 bp *Rsr*II-*Aat*II fragment of the synthetic KPI gene contained in pTW113 with a pair of annealed oligonucleotides which encode specific codons mutated from the wild-type KPI(-4→57) sequence. In the following Examples the convention used for designating the amino substituents in the KPI variants indicates first the single letter code for the amino acid found in wild-type KPI, followed by the position of the residue using the numbering convention described *supra*, followed by the code for the replacement amino acid. Thus, for example, M15R indicates that the methionine residue at position 15 is replaced by an arginine.

A. *Construction of pTW6165*

The strategy for constructing pTW6165 is shown in Figure 9. Plasmid pTW113 was digested with *Rsr*II and *Aat*II, and the larger of the two resulting fragments was isolated. An oligonucleotide pair (812 + 813) was phosphorylated, annealed and gel-purified as described above.

812 (SEQ ID NO:25):
GTCCGTGCCGTGCAGCTATCTGGCGCTGGTACTTTGACGT

813 (SEQ ID NO:26): CAAAGTACCAGCGCCAGATAGCTGCACGGCACG

The annealed oligonucleotides were ligated into the *Rsr*II and *Aat*II-digested pTW113, and the ligation product was used to transform *E. coli* strain MC1061. Transformed colonies were selected by ampicillin resistance. The resulting plasmid, pTW6165, encodes the 445 bp synthetic gene for the α-factor-KPI(-4→57; M15A, S17W) fusion. See Figure 10.

B. Construction of pTW6166, pTW6175, pBG028, pTW6183, pTW6184, pTW6185, pTW6173, pTW6174.

Construction of the following KPI(-4→57) variants was accomplished exactly as outlined for pTW6165. The oligonucleotides utilized for each construct are denoted below, and the sequences of annealed oligonucleotide pairs are shown in Figure 11. Figures 12-19 show the synthetic genes for the α-factor fusions with each KPI(-4→57) variant.

pTW6166: KPI(-4→57; M15A, S17Y) — See Figure 12

814 (SEQ ID NO:27):
GTCCGTGCCGTGCAGCTATCTACCGCTGGTACTTTGACGT

815 (SEQ ID NO:28): CAAAGTACCAGCGGTAGATAGCTGCACGGCACG

pTW6175: KPI(-4→57; M15L, S17F) — See Figure 13

867 (SEQ ID NO:29):
GTCCGTGCCGTGCATTGATCTTCCGCTGGTACTTTGACGT

868 (SEQ ID NO:30): CAAAGTACCAGCGGAAGATCAATGCACGGCACG

pBG028: KPI(-4→57; M15L, S17Y) — See Figure 14

1493 (SEQ ID NO:31):
GTCCGTGCCGTGCTTTGATCTACCGCTGGTACTTTGACGT

1494 (SEQ ID NO:32): CAAAGTACCAGCGGTAGATCAAAGCACGGCACG

pTW6183: KPI(-4→57; I16H, S17F) — See Figure 15

925 (SEQ ID NO:33):
GTCCGTGCCGTGCAATGCACTTCCGCTGGTACTTTGACGT

926 (SEQ ID NO:34): CAAAGTACCAGCGGAAGTGCATTGCACGGCACG

pTW6184: KPI(-4→57; I16H, S17Y) — See Figure 16

927 (SEQ ID NO:35):
GTCCGTGCCGTGCAATGCACTACCGCTGGTACTTTGACGT

928 (SEQ ID NO:36): CAAAGTACCAGCGGTAGTGCATTGCACGGCACG

pTW6185: KPI(-4→57; I16H, S17W) — See Figure 17

929 (SEQ ID NO:37):
GTCCGTGCCGTGCAATGCACTGGCGCTGGTACTTTGACGT

930 (SEQ ID NO:38): CAAAGTACCAGCGCCAGTGCATTGCACGGCACG

pTW6173: KPI(-4→57; M15A, I16H) — See Figure 18

863 (SEQ ID NO:39):
GTCCGTGCCGTGCAGCTCACTCCCGCTGGTACTTTGACGT

864 (SEQ ID NO:40): CAAAGTACCAGCGGGAGTGAGCTGCACGGCACG

pTW6174: KPI(-4→57; M15L, I16H) — See Figure 19

865 (SEQ ID NO:41):
GTCCGTGCCGTGCATTGCACTCCCGCTGGTACTTTGACGT

866 (SEQ ID NO:42): CAAAGTACCAGCGGGAGTGCAATGCACGGCACG

C. Transformation of yeast with expression vectors

Yeast strain ABL115 was transformed by electroporation exactly according to the protocol described for transformation by pTW113.

D. Induction of transformed yeast strains, purification of KPI(-4→57) variants.

Cultures of yeast strains were grown and induced, and recombinant secreted KPI(-4→57) variants were purified according to the procedure described for KPI(-4→57). The amino acid sequences of KPI(-4→57) variants are shown in Figures 20-29.

Example 3. Identification of KPI (-4→57; M15A, S17F) DD185 by phage display.

A. Construction of vector pSP26:Amp:F1

The construction of pSP26:*Amp*:F1 is outlined in Figure 30. Vector pSP26:*Amp*:F1 contributes the basic plasmid backbone for the construction of the phage display vector for the *pho*A:KPI fusion, PDW1 #14. pSP26:*Amp*:F1 contains a low-copy number origin of replication, the ampicillin-resistance gene (*Amp*) and the F1 origin for production of single-stranded phagemid DNA.

The ampicillin-resistance gene (*Amp*) was generated through polymerase chain reaction (PCR) amplification from the plasmid genome of PUC19 using oligonucleotides 176 and 177.

176 (SEQ ID NO:43):

GCCATCGATGGTTTCTTAAGCGTCAGGTGGCACTTTTC

177 (SEQ ID NO:44):

GCGCCAATTCTTGGTCTACGGGGTCTGACGCTCAGTGGAACGAA

The PCR amplification of *Amp* was done according to standard techniques, using Taq polymerase (Perkin-Elmer Cetus, Norwalk, CT). Amplification from plasmid pUC19 with these oligonucleotides yielded a fragment of 1159 bp, containing *Pfl*MI and *Cla*I restriction sites. The PCR product was digested with *Pfl*MI and *Cla*I and purified by agarose gel electrophoresis in 3% NuSieve Agarose (FMC Corp.). Bacterial expression vector pSP26 (*supra*) was digested with *Pfl*MI and *Cla*I and the larger vector fragment was purified. The *Pfl*MI-*Cla*I PCR fragment was ligated into the previously digested pSP26 containing the *Amp* gene. The ligation product was used to transform *E. coli* strain MC1061 and colonies were

selected by ampicillin resistance. The resulting plasmid is denoted pSP26:*Amp*.

The F1 origin of replication from the mammalian expression vector pcDNAII (Invitrogen) was isolated in a 692 bp *Ear*I fragment. Plasmid pcDNAII was digested with *Ear*I and the resulting 692 bp fragment purified by agarose gel electrophoresis. *Ear*I-*Not*I adapters were added to the 692 bp *Ear*I fragment by ligation of two annealed oligonucleotide pairs, 179 + 180 and 181 + 182. The oligo pairs were annealed as described above.

179 (SEQ ID NO:45): GGCCGCTCTTCC
180 (SEQ ID NO:46): AAAGGAAGAGC
181 (SEQ ID NO:47): CTAGAATTGC
182 (SEQ ID NO:48): GGCCGCAATTC

The oligonucleotide-ligated fragment was then ligated into the single *Not*I site of PSP26:*Amp* to yield the vector pSP26:*Amp*:F1.

B. Construction of vector pgIII

The construction of pgIII is outlined in Figure 31. The portion of the phage geneIII protein gene contained by the PDW1 #14 phagemid vector was originally obtained as a PCR amplification product from vector m13mp8. A portion of m13mp8 geneIII encoding the carboxyl-terminal 158 amino acid residues of the geneIII product was isolated by PCR amplification of m13mp8 nucleotide residues 2307-2781 using PCR oligos 6162 and 6160.

6162 (SEQ ID NO:49): GCCGGATCCGCTATTTCCGGTGGTGGCTCTGGTTCC
6160 (SEQ ID NO:50): GCCAAGCTTATTAAGACTCCTTATTACGCAG

The PCR oligos contain *Bam*HI and *Hin*dIII restriction recognition sites such that PCR from m13mp8 plasmid DNA with the oligo pair yielded a 490 bp *Bam*HI-*Hin*dIII fragment encoding the appropriate portion of geneIII. The PCR product was ligated between the *Bam*HI and *Hin*dIII

sites within the polylinker of PUC19 to yield plasmid pgIII.

C. Construction of pPhoA:KPI:gIII

Construction of p*Pho*A:KPI:gIII is outlined in Figure 32. A portion of the *pho*A signal sequence and KPI fusion encoded by the phage display vector PDW1 #14 originates with p*Pho*A:KPI:gIII. The 237 bp *Nde*I-*Hin*dIII fragment of pTW10:KPI encoding the entire *pho*A:KPI (1→57) fusion was isolated by preparative agarose gel electrophoresis, and inserted between the *Nde*I and *Hin*dIII sites of pUC19 to yield plasmid p*Pho*A:KPI. The 490 bp *Bam*HI-*Hin*dIII fragment of pgIII encoding the C-terminal portion of the geneIII product was then isolated and ligated between the *Bam*HI and *Hin*dIII sites of p*Pho*A:KPI to yield vector p*Pho*a:KPI:gIII. The p*Pho*A:KPI:gIII vector encodes a 236 amino acid residue fusion of the *pho*A signal peptide, KPI (1→57) and the carboxyl-terminal portion of the geneIII product.

D. Construction of pLG1

Construction of pLG1 is illustrated in Figure 33. The exact geneIII sequences contained in vector PDW1 #14 originate with phage display vector pLG1. A modified geneIII segment was generated by PCR amplification of the geneIII region from pgIII using PCR oligonucleotides 6308 and 6305.

6308 (SEQ ID NO:51):
AGCTCCGATCTAGGATCCGGTGGTGGCTCTGGTTCCGGT
6305 (SEQ ID NO:52): GCAGCGGCCGTTAAGCTTATTAAGACTCCT

PCR amplification from pgIII with these oligonucleotides yielded a 481 bp *Bam*HI-*Hin*dIII fragment encoding a geneIII product shortened by 3 amino acid residues at the amino-terminal portion of the segment of the geneIII fragment encoded by pgIII. A 161 bp *Nde*I-*Bam*HI fragment was generated by PCR amplification from

bacterial expression plasmid pTHW05 using oligonucleotides 6306 and 6307.

6306 (SEQ ID NO:53): GATCCTTGTGTCCATATGAAACAAAGC

6307 (SEQ ID NO:54):
CACGTCGGTCGAGGATCCCTAACCACGGCCTTTAACCAG

The 161 bp *Nde*I-*Bam*HI fragment and the 481 bp *Bam*HI-*Hin*dIII fragment were gel-purified, and then ligated in a three-way ligation into PTW10 which had previously been digested with *Nde*I and *Hin*dIII. The resulting plasmid pLG1 encodes a *pho*A signal peptide-insert-geneIII fusion for phage display purposes.

E. Construction of pAL51

Construction of pAL51 is illustrated in Figure 34. Vector pAL51 contains the geneIII sequences of pLG1 which are to be incorporated in vector pDW1 #14.

A 1693 bp fragment of plasmid pBR322 was isolated, extending from the *Bam*HI site at nucleotide 375 to the *Pvu*II site at position 2064. Plasmid pLG1 was digested with *Asp*718I and *Bam*HI, removing an 87 bp fragment. The overhanging *Asp*718I end was blunted by treatment with Klenow fragment, and the *Pvu*II-*Bam*HI fragment isolated from pBR322 was ligated into this vector, resulting in the insertion of a 1693 bp "stuffer" region between the *Asp*718I and *Bam*HI sites. The 78 bp *Nde*I-*Asp*718I region of the resulting plasmid was removed and replaced with the annealed oligo pair 6512 + 6513.

6512 (SEQ ID NO:55): TATGAAACAAAGCACTATTGCACTGGCACT
CTTACCGTTACTGTTTACCCCGGTGACCAAAGCCCACGCTGAAG

6513 (SEQ ID NO:56): GTACCTTCAGCGTGGGCTTTGGTCACCGGG
GTAAACAGTAACGGTAAGAGTGCCAGTGCAATAGTGCTTTGTTTCA

The newly created 74 bp *Nde*I-*Asp*718I fragment encodes the *pho*A signal peptide, and contains a *Bst*EII cloning site. The resulting plasmid is denoted pAL51.

F. Construction of pAL53

Construction of pAL53 is outlined in Figure 35. Plasmid pAL53 contributes most of the vector sequence of pDW1 #14, including the basic vector backbone with *Amp* gene, F1 origin, low copy number origin of replication, geneIII segment, *phoA* promotor and *phoA* signal sequence.

Plasmid pAL51 was digested with *NdeI* and *HindIII* and the resulting 2248 bp *NdeI-HindIII* fragment encoding the *phoA* signal peptide, stuffer region and geneIII region was isolated by preparative agarose gel electrophoresis. The *NdeI-HindIII* fragment was ligated into plasmid pSP26:*Amp*:F1 between the *NdeI* and *HindIII* sites, resulting in plasmid pAL52.

The *phoA* promoter region and signal peptide was generated by amplification of a portion of the *E. coli* genome by PCR, using oligonucleotide primers 405 and 406.

405 (SEQ ID NO:57): CCGGACGCGTGGAGATTATCGTCACTG
406 (SEQ ID NO:58): GCTTTGGTCACCGGGGTAAACAGTAACGG

The resulting PCR product is a 332 bp *MluI-BstEII* fragment which contains the *phoA* promoter region and signal peptide sequence. This fragment was used to replace the 148 bp *MluI-BstEII* segment of PAL52, resulting in vector pAL53.

G. Construction of pSP26:Amp:F1:PhoA:KPI:gIII

Construction of pSP26:*Amp*:F1:*PhoA*:KPI:gIII is illustrated in Figure 36. This particular vector is the source of the KPI coding sequence found in vector pDW1 #14. Plasmid p*Phoa*:KPI:gIII was digested with *NdeI* and *HindIII*, and the resulting 714 bp *NdeI-HindIII* fragment was purified, and then inserted into vector pSP26:*Amp*:F1 between the *NdeI* and *HindIII* sites. The resulting plasmid is denoted pSP26:*Amp*:F1:*PhoA*:KPI:gIII.

H. Construction of pDW1 #14

Construction of pDW1 #14 is illustrated in Figure 37. The sequences encoding KPI were amplified from plasmid

pSP26:*Amp*:F1:*Pho*A:KPI:gIII by PCR, using oligonucleotide primers 424 and 425.

424 (SEQ ID NO:54):
CTGTTTACCCCGGTGACCAAAGCCGAGGTGTGCTCTGAACAA

425 (SEQ ID NO:55):
AATAGCGGATCCGCACACTGCCATGCAGTACTCTTC

The resulting 172 bp *Bst*EII-*Bam*HI fragment encodes most of KPI (1→55). This fragment was used to replace the stuffer region in pAL53 between the *Bst*EII and *Bam*HI sites. The resulting plasmid, PDW1 #14, is the parent KPI phage display vector for preparation of randomized KPI phage libraries. The coding region for the *pho*A-KPI (1→55)-geneIII fusion is shown in Figure 38.

I. Construction of pDW1 14-2

Construction of pDW1 14-2 is illustrated in Figure 39. The first step in the construction of the KPI phage libraries in pDW1 #14 was the replacement of the *Age*I-*Bam*HI fragment within the KPI coding sequence with a stuffer fragment. This greatly aids in preparation of randomized KPI libraries which are substantially free of contamination of phagemid genomes encoding wild-type KPI sequence.

Plasmid pDW1 #14 was digested with *Age*I and *Bam*HI, and the 135 bp *Age*I-*Bam*HI fragment encoding KPI was discarded. A stuffer fragment was created by PCR amplification of a portion of the PBR322 *Tet* gene, extending from the *Bam*HI site at nucleotide 375 to nucleotide 1284, using oligo primers 266 and 252.

266 (SEQ ID NO:61): GCTTTAAACCGGTAGGTGGCCCGGCTCCATGCACC

252 (SEQ ID NO:62):
CGAATTCACCGGTGTCATCCTCGGCACCGTCACCCT

The resulting 894 bp *Age*I-*Bam*HI stuffer fragment was then inserted into the *Age*I/*Bam*HI-digested pDW1 #14 to yield the phagemid vector pDW1 14-2. This vector

was the starting point for construction of the randomized KPI libraries.

J. Construction of KPI Library 16-19

Construction of KPI Library 16-19 is outlined in Figure 40. Library 16-19 was constructed to display KPI-geneIII fusions in which amino acid positions Ala^{14}, Met^{15}, Ile^{16} and Ser^{17} are randomized. For preparation of the library, plasmid pDW1 14-2 was digested with *AgeI* and *BamHI* to remove the stuffer region, and the resulting vector was purified by preparative agarose gel electrophoresis. Plasmid pDW1 #14 was used as template in a PCR amplification of the KPI region extending from the *AgeI* site to the *BamHI* site. The oligonucleotide primers used were 544 and 551.

544 (SEQ ID NO:63):
GGGCTGAGACCGGTCCGTGCCGT(NNS)$_4$CGCTGGTACTTTGACGTC

551 (SEQ ID NO:64): GGAATAGCGGATCCGCACACTGCCATGCAG

Oligonucleotide primer 544 contains four randomized codons of the sequence NNS, where N represents equal mixtures of A/G/C/T and S an equal mixture of G or C. Each NNS codon thus encodes all 20 amino acids plus a single possible stop codon, in 32 different DNA sequences. PCR amplification from the wild-type KPI gene resulted in the production of a mixture of 135 bp *AgeI*-*BamHI* fragments all containing different sequences in the randomized region. The PCR product was purified by preparative agarose gel electrophoresis and ligated into the *AgeI*/*BamHI* digested PDW1 14-2 vector. The ligation mixture was used to transform *E. coli* Top10F' cells (Invitrogen) by electroporation according to the manufacturer's directions. The resulting Library 16-19 contained approximately 400,000 independent clones. The potential size of the library, based upon the degeneracy of the priming PCR oligo #544 was 1,048,576 members. The expression unit encoded by the members of Library 16-19 is shown in Figure 41.

K. Selection of Library 16-19 with human plasma kallikrein

KPI phage were prepared and amplified by infecting transformed cells with M13KO7 helper phage as described by Matthews *et al.*, *Science* 260:1113 (1993). Human plasma kallikrein (Enzyme Research Laboratories, South Bend, IN), was coupled to Sepharose 6B resin. Prior to phage binding, the immobilized kallikrein resin was washed three times with 0.5 ml assay buffer (AB = 100mM Tris-HCl, pH 7.5, 0.5M NaCl, 5mM each of KCl, $CaCl_2$, $MgCl_2$, 0.1% gelatin, and 0.05% Triton X-100). Approximately $5x10^9$ phage particles of the amplified Library 16-19 in PBS, pH 7.5, containing 300mM NaCl and 0.1% gelatin, were bound to 50 µl kallikrein resin containing 15 pmoles of active human plasma kallikrein in a total volume of 250 µl. Phage were allowed to bind for 4 h at room temperature, with rocking. Unbound phage were removed by washing the kallikrein resin three times in 0.5 ml AB. Bound phage were eluted sequentially by successive 5 minute washes: 0.5 ml 50mM sodium citrate, pH 6.0, 150mM NaCl; 0.5 ml 50mM sodium citrate, pH 4.0, 150mM NaCl; and 0.5 ml 50mM glycine, pH 2.0, 150mM NaCl. Eluted phage were neutralized immediately and phagemids from the pH 2.0 elution were titered and amplified for reselection. After three rounds of selection on kallikrein-Sepharose, phagemid DNA was isolated from 22 individual colonies and subjected to DNA sequence analysis.

The most frequently occurring randomized KPI region encoded (SEQ ID NO:65): Ala^{14}-Ala^{15}-Ile^{16}-Phe^{17}. The *pho*A-KPI-geneIII region encoded by this class of selected KPI phage is shown in Figure 42. The KPI variant encoded by these phagemids is denoted KPI (1→55; M15A, S17F).

L. Construction of pDD185 KPI (-4→57; M15A, S17F)

Figure 43 outlines the construction of pDD185 KPI (-4→57; M15A, S17F). The sequences encoding KPI (1→55; M15A, S17F) were moved from one phagemid vector, pDW1

(16-19) 185, to the yeast expression vector so that the KPI variant could be purified and tested.

Plasmid pTW113 encoding wild-type KPI (-4→57) was digested with *Age*I and *Bam*HI and the 135 bp *Age*I-*Bam*HI fragment was discarded. The 135 bp *Age*I-*Bam*HI fragment of pDW1 (16-19) 185 was isolated and ligated into the yeast vector to yield plasmid pDD185, encoding α-factor fused to KPI (-4→57; M15A, S17F). See Figure 44.

M. Purification of KPI (-4→57; M15A, S17F) pDD185

Transformation of yeast strain ABL115 with pDD185, induction of yeast cultures, and purification of KPI (-4→57; M15A, S17F) pDD185 was accomplished as described for the other KPI variants.

N. Construction of KPI Library 6 — M15A, with residues 14, 16-18 random.

Library 6 was constructed to display KPI-geneIII fusions in which amino acid positions Ala^{14}, Ile^{16}, Ser^{17} and Arg^{18} are randomized, but position 15 was held constant as Ala. For preparation of the library, plasmid pDW1 #14 was used as template in a PCR amplification of the KPI region extending from the *Age*I site to the *Bam*HI site. The oligonucleotide primers used were 551 and 1003.

1003 (SEQ ID NO:66):
GCTGAGACCGGTCCGTGCCGTNNSGCA$(NNS)_3$TGGTACTTTGACGTC

551 (SEQ ID NO:64): GGAATAGCGGATCCGCACACTGCCATGCAG

Oligonucleotide primer 1003 contained four randomized codons of the sequence NNS, where N represents equal mixtures of A/G/C/T and S an equal mixture of G or C. Each NNS codon thus encodes all 20 amino acids plus a single possible stop, in 32 different DNA sequences. PCR amplification from the wild-type KPI gene resulted in the production of a mixture of 135 bp *Age*I-*Bam*HI fragments all containing different sequences in the randomized region. The PCR product was phenol extracted, ethanol

precipitated, digested with *Bam*HI and purified by preparative agarose gel electrophoresis. Plasmid pDW1 14-2 was digested with *Bam*HI, phenol extracted and ethanol precipitated. The insert was ligated at high molar ratio to the vector which was then digested with *Age*I to remove the stuffer region. The vector containing the insert was purified by agarose gel electrophoresis and recircularized. The resulting library contains approximately $5x10^6$ independent clones.

O. Construction of KPI Library 7 — residues 14-18 random.

Library 7 was constructed to display KPI-geneIII fusions in which amino acid positions Ala^{14}, Met^{15}, Ile^{16}, Ser^{17} and Arg^{18} are randomized. For preparation of the library, plasmid pDW1 #14 was used as template in a PCR amplification of the KPI region extending from the *Age*I site to the *Bam*HI site. The oligonucleotide primers used were 551 and 1179.

1179 (SEQ ID NO:67):
GCTGAGACCGGTCCGTGCCGT(NNS)$_5$TGGTACTTTGACGTC

551 SEQ ID NO:64): GGAATAGCGGATCCGCACACTGCCATGCAG

Oligonucleotide primer 1179 contains five randomized codons of the sequence NNS, where N represents equal mixtures of A/G/C/T and S an equal mixture of G or C. Each NNS codon thus encoded all 20 amino acids plus a single possible stop, in 32 different DNA sequences. PCR amplification from the wild-type KPI gene resulted in the production of a mixture of 135 bp *Age*I-*Bam*HI fragments all containing different sequences in the randomized region. The PCR product was phenol extracted, ethanol precipitated, digested with *Bam*HI and purified by preparative agarose gel electrophoresis. Plasmid pDW1 14-2 was digested with *Bam*HI, phenol extracted and ethanol precipitated. The insert was ligated at high molar ratio to the vector which was then digested with

*Age*I to remove the stuffer region. The vector containing the insert was purified by agarose gel electrophoresis and recircularized. The resulting library contains approximately $1x10^7$ independent clones.

P. Selection of Libraries 6 & 7 with human factor XIIa

KPI phage were prepared and amplified by infecting transformed cells with M13K07 helper phage (Matthews and Wells, 1993). Human factor XIIa (Enzyme Research Laboratories, South Bend, IN), was biotinylated as follows. Factor XIIa (0.5 mg) in 5mM sodium acetate pH 8.3 was incubated with Biotin Ester (Zymed) at room temperature for 1.5 h, then buffer-exchanged into assay buffer (AB). Approximately $1x10^{10}$ phage particles of each amplified Library 6 or 7 in PBS, pH 7.5, containing 300mM NaCl and 0.1% gelatin, were incubated with 50 pmoles of active biotinylated human factor XIIa in a total volume of 200 µl. Phage were allowed to bind for 2 h at room temperature, with rocking. Following the binding period, 100 µl Strepavidin Magnetic Particles (Boehringer Mannheim) were added to the mixture and incubated at room temperature for 30 minutes. Separation of magnetic particles from the supernatant and wash/elution buffers was carried out using MPC-E-1 Neodymium-iron-boron permanent magnets (Dynal). Unbound phage were removed by washing the magnetically bound biotinylated XIIa-phage complexes three times with 0.5 ml AB. Bound phage were eluted sequentially by successive 5 minute washes: 0.5 ml 50mM sodium citrate, pH 6.0, 150mM NaCl; 0.5 ml 50mM sodium citrate, pH 4.0, 150mM NaCl; and 0.5 ml 50mM glycine, pH 2.0, 150mM NaCl. Eluted phage were neutralized immediately and phagemids from the pH 2.0 elution were titered and amplified for reselection. After 3 or 4 rounds of selection with factor XIIa, phagemid DNA was isolated from individual colonies and subjected to DNA sequence analysis.

Sequences in the randomized regions were compared with one another to identify consensus sequences

appearing more than once. From Library 6 a phagemid was identified which encoded M15L, S17Y, R18H. From Library 7 a phagemid was identified which encoded M15A, S17Y, R18H.

Q. Construction of pBG015 KPI (-4→57; M15L, S17Y, R18H), pBG022 (-4→57; M15A, S17Y, R18H)

The sequences encoding KPI (1→55; M15L, S17Y, R18H) and KPI (1→55; M17A, S17Y, R18H) were moved from the phagemid vectors to the yeast expression vector so that the KPI variant could be purified and tested.

Plasmid pTW113 encoding wild-type KPI (-4→57) was digested with *Age*I and *Bam*HI and the 135 bp *Age*I-*Bam*HI fragment was discarded. The 135 bp *Age*I-*Bam*HI fragment of the phagemid vectors were isolated and ligated into the yeast vector to yield plasmids pBG015 and pBG022, encoding alpha-factor fused to KPI (-4→57; M15L, S17Y, R18H), and KPI (-4→57; M15A, S17Y, R18H), respectively.

R. Construction of pBG029 KPI (-4→57, T9V, M15L, S17Y, R18H)

Plasmid pBG015 was digested with *Xba*I and *Rsr*II, and the larger of the two resulting fragments was isolated. An oligonucleotide pair (1593 + 1642) was phosphorylated, annealed and gel-purified as described previously.

1593 (SEQ ID NO:68): CTAGATAAAAGAGAGGTTGTTAGAGAGGTG
TGCTCTGAACAAGCTGAGGTTG

1642 (SEQ ID NO:69):
GACCAACCTCAGCTTGTTCAGAGCACACCTCTCTAACAACCTCTCTTTTAT

The annealed oligonucleotides were ligated into the *Xba*I and *Rsr*II-digested pBG015, and the ligation product was used to transform *E. coli* strain MC1061 to ampicillin resistance. The resulting plasmid pBG029, encodes the 445 bp synthetic gene for the alpha-factor-KPI (-4→57; T9V, M15L, S17F, R18H) fusion.

S. Construction of pBG033 KPI (-4→57; T9V, M15A, S17Y, R18H)

Plasmid pBG022 was digested with *Xba*I and *Rsr*II, and the larger of the two resulting fragments was isolated. An oligonucleotide pair (1593 + 1642) was phosphorylated, annealed and gel-purified as described previously. The annealed oligonucleotides were ligated into the *Xba*I and *Rsr*II-digested pBG022, and the ligation product was used to transform *E. coli* strain MC1061 to ampicillin resistance. The resulting plasmid pBG033, encodes the 445 bp synthetic gene for the alpha-factor-KPI (-4→57; T9V, M15A, S17F, R18H) fusion.

T. Selection of Library 16-19 with human factor Xa

KPI phage were prepared and amplified by infecting transformed cells with M13K07 helper phage (Matthews and Wells, 1993). Human factor Xa (Haematologic Technologies, Inc., Essex Junction, VT) was coupled to Sepharose 6B resin. Prior to phage binding, the immobilized Xa resin was washed three times with 0.5 ml assay buffer (AB = 100mM Tris-HCl, pH 7.5, 0.5M NaCl, 5mM each of KCl, $CaCl_2$, $MgCl_2$, 0.1% gelatin, and 0.05% Triton X-100). Approximately $4x10^{10}$ phage particles of the amplified Library 16-19 in PBS, pH 7.5, containing 300mM NaCl and 0.1% gelatin, were bound to 50 µl Xa resin in a total volume of 250 µl. Phage were allowed to bind for 4 h at room temperature, with rocking. Unbound phage were removed by washing the Xa resin three times in 0.5 ml AB. Bound phage were eluted sequentially by successive 5 minute washes: 0.5 ml 50mM sodium citrate, pH 6.0, 150mM NaCl; 0.5 ml 50mM sodium citrate, pH 4.0 150mM NaCl; and 0.5 ml 50mM glycine, pH 2.0, 150mM NaCl. Eluted phage were neutralized immediately and phagemids from the pH 2.0 elution were titered and amplified for reselection. After three rounds of selection on Xa-Sepharose, phagemid DNA was isolated and subjected to DNA sequence analysis.

Sequences in the randomized Ala^{14}-Ser^{17} region were compared with one another to identify consensus sequences appearing more than once. A phagemid was identified which encoded KPI (1→55; M15L, I16F, S17K).

U. Construction of pDD131 KPI (-4→57; M15L, I16F, S17K)

The sequences encoding KPI (1→55; M15L, I16F, S17K) were moved from the phagemid vector to the yeast expression vector so that the KPI variant could be purified and tested.

Plasmid pTW113 encoding wild-type KPI (-4→57) was digested with *Age*I and *Bam*HI and the 135 bp *Age*I-*Bam*HI fragment was discarded. The 135 bp *Age*I-*Bam*HI fragment of the phagemid vector was isolated and ligated into the yeast vector to yield plasmid pDD131, encoding alpha-factor fused to KPI (-4→57; M15L, I16F, S17K).

V. Construction of pDD134 KPI (-4→57; M15L, I16F, S17K, G37Y)

Plasmid pDD131 was digested with *Aat*I and *Bam*HI, and the larger of the two resulting fragments was isolated. An oligonucleotide pair (738 + 739) was phosphorylated, annealed and gel-purified as described previously.

738 (SEQ ID NO. 70):
CACTGAAGGTAAGTGCGCTCCATTCTTTTACGGCGGTTGCTACGGCAA
CCGTAACAACTTTGACACTGAAGAGTACTGCATGGCAGTGTGCG

739 (SEQ ID NO:71):
GATCCGCACACTGCCATGCAGTACTCTTCAGTGTCAAAGTTGTTACGGTTGC
CGTAGCAACCGCCGTAAAAGAATGGAGCGCACTTACCTTCAGTGACGT

The annealed oligonucleotides were ligated into the *Aat*I and *Bam*HI-digested pDD131, and the ligation product was used to transform *E. coli* strain MC1061 to ampicillin resistance. The resulting plasmid pDD134, encodes the 445 bp synthetic gene for the alpha-factor-KPI (-4→57; M15L, I16F, S17K, G37Y) fusion.

W. Construction of pDD135 KPI (-4→57; M15L, I16F, S17K, G37L)

Plasmid pDD131 was digested with *Aat*II and *Bam*HI, and the larger of the two resulting fragments was isolated. An oligonucleotide pair (724 + 725) was phosphorylated, annealed and gel-purified as described previously.

724 (SEQ ID NO:72):
CACTGAAGGTAAGTGCGCTCCATTCTTTTACGGCGGTTGCTTGGGCAACCGTAAC
AACTTTGACACTGAAGAGTACTGCATGGCAGTGTGCG

725 (SEQ ID NO:73)
GATCCGCACACTGCCATGCAGTACTCTTCAGTGTCAAAGTTGTTACGGTTGCCCA
AGCAACCGCCGTAAAAGAATGGAGCGCACTTACCTTCAGTGACGT

The annealed oligonucleotides were ligated into the *Aat*II and *Bam*HI-digested pDD131, and the ligation product was used to transform *E. coli* strain MC1061 to ampicillin resistance. The resulting plasmid pDD135, encodes the 445 bp synthetic gene for the alpha-factor-KPI (-4→57; M15L, I16F, S17K, G37L) fusion.

Example 4. Kinetic analysis of KPI(-4→57) variants

The concentrations of active human plasma kallikrein, factor XIIa, and trypsin were determined by titration with p-nitrophenyl p'-guanidinobenzoate as described by Bender *et al.*, *supra*, and Chase *et al.*, *Biochem. Biophys. Res. Commun.* 29:508 (1967). Accurate concentrations of active KPI(-4→57) inhibitors were determined by titration of the activity of a known amount of active-site-titrated trypsin. For testing against kallikrein and trypsin, each KPI(-4→57) variant (0.5 to 100nM) was incubated with protease in low-binding 96-well microtiter plates at 30°C for 15-25 min, in 100mM Tris-HCl, pH 7.5, with 500mM NaCl, 5mM KCl, 5mM CaCl2, 5mM MgCl2, 0.1% Difco gelatin, and 0.05% Triton X-100. Chromogenic synthetic substrate was then be added, and initial rates at 30°C recorded by the SOFTmax kinetics program via a THERMOmax microplate reader (Molecular Devices Corp., Menlo Park, CA). The substrates used were N-α-benzoyl-L-Arg p-nitroanilide

nitroanilide (0.3mM) for plasma kallikrein (1nM). The Enzfitter (Elsevier) program was used both to plot fractional activity (i.e., activity with inhibitor, divided by activity without inhibitor), a, versus total concentration of inhibitor, I_t, and to calculate the dissociation constant of the inhibitor (K_i) by fitting the curve to the following equation:

$$a=1-\frac{[E]_t+[I]_t+K_i-\sqrt{([E]_t+[I]_t+K_i)^2-4[E]_t[I]_t}}{2[E]_t}$$

The K_is determined for purified KPI variants are shown in Figure 45. The most potent variant, KPI (-4→57; M15A, S17F) DD185 is 115-fold more potent as a human kallikrein inhibitor than wild-type KPI (-4→57). The least potent variant, KPI (-4→57; I16H, S17W) TW6185 is still 35-fold more potent than wild-type KPI.

For testing against factor XIIa, essentially the same reaction conditions were used, except that the substrate was N-benzoyl-Ile-Glu-Gly-Arg p-nitroaniline hydrochloride and its methyl ester (obtained from Pharmacia Hepar, Franklin, OH), and corn trypsin inhibitor (Enzyme Research Laboratories, South Bend, IN) was used as a control inhibitor. Factor XIIa was also obtained from Enzyme Research Laboratories.

Various data for inhibition of the serine proteases of interest kallikrein, plasmin, and factors Xa, XIa, and XIIa by a series of KPI variants are given in Figure 46. The results indicate that KPI variants can be produced that can bind to and preferably inhibit the activity of serine proteases. The results also indicate that the peptides of the invention may exhibit the preferable more potent and specific inhibition of one or more serine proteases of interest.

Example 5. Effect of KPI variant KPI185-1 on postoperative bleeding

A randomized, double-blinded study using an acute porcine cardiopulmonary bypass (CPB) model was used to investigate the effect of KPI185-1 on postoperative bleeding. Sixteen pigs (55-65 kg) underwent 60 minutes of hypothermic (28°C) open-chest CPB with 30 minutes of cardioplegic cardiac arrest. Pigs were randomized against a control solution of physiological saline (NS; n=8) or KPI-185 (n=8) groups. During aortic cross-clamping, the tricuspid valve was inspected through an atriotomy which was subsequently repaired. Following reversal of heparin with protamine, dilateral thoracostomy tubes were placed and shed blood collected for 3 hours. Shed blood volume and hemoglobin (Hgb) loss were calculated from total chest tube output and residual intrathoracic blood at time of sacrifice.

Total blood loss was significantly reduced in the KPI185-1 group (245.75 ± 66.24 ml vs. 344.25 ± 63.97 ml, p=0.009). In addition, there was a marked reduction in total Hgb loss in the treatment group (13.59 ± 4.26 gm vs. 23.61 ± 4.69 gm, p=0.0005). Thoracostomy drainage Hgb was significantly increased at 30 and 60 minutes in the control group [6.89 ± 1.44 vs. 4.41 ± 1.45 gm/dl (p=0.004) and 7.6 ± 1.03 vs. 5.26 ± 1.04 gm/dl (p=0.0002), respectively]. Preoperative and post-CPB hematocrits were not statistically different between the groups. These results are shown in graphical form in Figures 47-50.

The invention has been disclosed broadly and illustrated in reference to representative embodiments described above. Those skilled in the art will recognize that various modifications can be made to the present invention without departing from the spirit and scope thereof.